UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

UROPATHOGENIC *PROTEUS MIRABILIS* AND *KLEBSIELLA PNEUMONIAE* IN COMPANION ANIMALS: MOLECULAR EPIDEMIOLOGY, ANTIMICROBIAL RESISTANCE AND ZOONOTIC POTENTIAL

CÁTIA FILIPA SARAIVA MARQUES

Orientadora: Professora Doutora Maria Constança Matias Ferreira Pomba

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Clínica

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Presidente: Professor Doutor Luís Filipe Lopes da Costa
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2019

This work is dedicated to my dad, my mom and Pedro.

I am here because of you…

and a little bit because of fadinha and pretinha for keeping my feet warm while writing.

With all my heart.

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"*It takes a village to raise a child*"
(proverb)

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Resumo

Proteus mirabilis e _Klebsiella pneumoniae_ uropatogénicos em animais de companhia: epidemiologia molecular, resistência aos antimicrobianos e potencial zoonótico

As infeções bacterianas do trato urinário (ITU) são frequentemente diagnosticadas em animais de companhia e no homem. O aumento da resistência e multirresistência aos antimicrobianos é um reconhecido problema na sociedade moderna que resulta na diminuição de opções terapêuticas. Com o crescente contacto entre os animais de companhia e o homem, a disseminação de bactérias resistentes a antimicrobianos criticamente importantes levanta também grandes preocupações de saúde pública.

Seguindo o modelo utilizado pelo *European Centre for Disease Prevention and Control* (ECDC) nos relatórios de resistência aos antimicrobianos de bactérias invasivas de origem humana, o primeiro estudo apresentado nesta tese pretendeu avaliar a etiologia e a distribuição geográfica da resistência aos antimicrobianos em bactérias (*n*= 22 256) isoladas de animais de companhia com ITU em 14 países Europeus. Este estudo mostrou que, em 2012-2013, a frequência de resistência aos antimicrobianos em *Escherichia coli* e *Proteus mirabilis* era significativamente superior nos países estudados do Sul da Europa (Portugal, Espanha, Itália e Grécia) quando comparada com os países estudados do Norte da Europa (Dinamarca e Suécia). O mesmo aconteceu quanto a *E. coli* e *P. mirabilis* multirresistente (MDR).

A elevada resistência aos antimicrobianos detetada em cães e gatos com ITU em Portugal (Lisboa) motivou a realização de um segundo estudo, retrospetivo, com o objetivo de avaliar a evolução temporal da resistência aos antimicrobianos, nesta região, ao longo de um período de 16 anos (1999-2014). Este estudo confirmou a ocorrência de um aumento significativo de resistência ao longo do tempo quanto à amoxicilina/clavulanato, cefalosporinas de terceira geração (C3G), trimetoprim/sulfametoxazol, fluoroquinolonas, gentamicina e tetraciclina ($P < 0,001$) em Enterobacteriaceae de animais de companhia com ITU. Adicionalmente, foi detetado um aumento significativo no isolamento de Enterobacteriaceae MDR ($P < 0,0001$). A resistência a C3G esteve associada a fenótipos de MDR e à presença de $bla_{\text{CTX-M-15}}$ e $bla_{\text{CMY-2}}$ em *E. coli* e de $bla_{\text{CMY-2}}$ em *P. mirabilis*. No que respeita a bactérias Gram-positivo, foi observado um aumento significativo na resistência à meticilina em *Staphylococcus pseudintermedius* ($P = 0,0069$). A caracterização por MLST de *E. coli*, *Staphylococcus* spp. e *Enterococcus* spp. positivos a mecanismos de resistência clinicamente relevantes revelou que os animais de companhia podem desenvolver ITUs causadas por bactérias pertencentes a linhagens clonais de elevado risco para o homem, nomeadamente, *E. coli* O25b:H4-B2-ST131, CC23 e ST648 resistente a C3G, *Staphylococcus aureus* meticilina-resistente do CC5, *Staphylococcus epidermidis* meticilina-resistente do CC5, *Enterococcus faecalis* do CC6 com

resistência à gentamicina em alto nível pela presença da enzima bifuncional e *Enterococcus faecium* do CC17 com resistência à ampicilina.

Ambos os estudos confirmaram a predominância de ITUs causadas por *E. coli* em cães e gatos. No entanto, algumas diferenças na etiologia da ITU foram detetadas, sendo as mais relevantes a frequência superior de *P. mirabilis* em cães e de *Enterococcus* spp. em gatos ($P < 0,0001$).

Klebsiella pneumoniae e *P. mirabilis* são importantes bactérias associadas a ITUs nosocomiais no homem. Embora *K. pneumoniae* seja menos frequente em animais de companhia, é a segunda bactéria mais frequente na ITU da mulher e está intimamente associada à disseminação de β-lactamases de espectro alargado (ESBL – *Extended-Spectrum Beta-Lactamases*) e carbapenemases. Os estudos epidemiológicos sobre *K. pneumoniae* e *P. mirabilis* em animais de companhia com ITU são escassos. No entanto, é essencial entender o papel dos cães e gatos com ITU na disseminação de *K. pneumoniae* e *P. mirabilis*, bem como a sua relevância numa perspetiva *One Health*.

Na segunda parte desta tese, constam dois estudos em que foi caracterizada a estrutura da população, a resistência aos antimicrobianos e a presença de genes de virulência em *K. pneumoniae* e *P. mirabilis* isolados de animais de companhia (*K. pneumoniae n*= 25; *P. mirabilis n*= 107) e humanos (*K. pneumoniae n*= 77; *P. mirabilis n*= 76) com ITU.

Os animais de companhia com ITU apresentaram um elevado número de *K. pneumoniae* MDR (80%, *n*= 20/25) e resistente a C3G (60%, *n*= 15/25). Em concordância com estudos Europeus anteriores, o gene $bla_{CTX\text{-}M\text{-}15}$ mostrou-se disseminado em *K. pneumoniae* resistente a 3GC tanto de origem em animais de companhia como de origem humana. Sessenta por cento (*n*=15/25) de *K. pneumoniae* isolada de animais de companhia com ITU em Portugal (Lisboa) pertenceu à linhagem clonal de elevado risco ST15. Outras linhagens clonais de elevado risco de *K. pneumoniae* isoladas de animais de companhia incluíram o ST11 (*n*= 1), ST37 (*n*= 1) e ST147 (*n*= 1), todos eles MDR e resistentes a C3G. *K. pneumoniae* resistente a C3G de origem humana mostrou ser mais diversificada, pertencendo a um maior número de linhagens clonais. Na análise de electroforese em gel de campo pulsado (PFGE – *Pulsed-Field Gel Electrophoresis*), a maioria das estirpes de *K. pneumoniae* ST15 de animais de companhia agrupou em dois *clusters* (C4 e C5; >80% de índice Dice/UPGMA, *Xba*I, 1,5% de tolerância), os quais incluíram algumas estirpes de origem humana. A linhagem clonal ST11 foi detetada num gato (*n*= 1) e num humano (*n*= 1); ambos MDR e com 81,1% de índice Dice/UPGMA. De notar que, duas estirpes pertencentes ao ST348, resistentes a 3GC e com 86,7% de índice Dice/UPGMA foram também isoladas de um gato e um humano. *K. pneumoniae* de ambas as origens apresentaram dois genótipos de virulência principais, nomeadamente *fimH-1*/*mrkD*/*entB*/*ycfM*/*kfu* e *fimH-1*/*mrkD*/*entB*/*ycfM*/*kpn*. A ilha de elevada patogenicidade yersiniabactina foi também comum em *K. pneumoniae* de animais de companhia (16%) e humanos (42,8%).

Como acima mencionado, a resistência às C3G em *P. mirabilis* de animais de companhia com ITU aumentou ao longo do tempo e esteve associada à presença de bla_{CMY-2}. Na análise de PFGE, um elevado número de *clusters* (43,6%, *n*= 17/39; >80% de índice Dice/UPGMA, *Not*I, 1,5% de tolerância) incluiu estirpes de *P. mirabilis* isoladas de animais de companhia e de humanos. É de notar que uma estirpe de *P. mirabilis* isolada de um cão foi 100% semelhante por PFGE (Dice/UPGMA), a uma estirpe isolada de um humano. Os genes de virulência testados (fimbria: *mrpA*, *pmfA*, *ucaA*; hemolisina: *HmpA*/*HmpB*) foram detetados na maioria das estirpes de ambas as origens (85% - 100%).

Ambos os estudos apresentados na segunda parte desta tese mostraram que *K. pneumoniae* e *P. mirabilis* de animais de companhia com ITU podem conter genes de resistência clinicamente importantes e que a maioria das estirpes de *K. pneumoniae* era MDR e pertencia a linhagens clonais de elevado risco para o Homem.

Durante a ITU, os animais de companhia apresentam uma elevada carga de bactérias uropatogénicas na urina pelo que poderão contribuir para a sua disseminação. O papel dos animais de companhia saudáveis como reservatórios de bactérias patogénicas é também uma preocupação de saúde pública. Estudos anteriores mostraram que estirpes de *E. coli* são partilhadas entre animais de companhia e humanos a viver juntos. Contudo, tanto quanto nos foi possível averiguar, tais estudos não foram realizados relativamente a *K. pneumoniae* e *P. mirabilis.* Para tal, na terceira parte desta tese, animais de companhia (*n*= 18 cães e *n*= 8 gatos) e humanos (*n*= 24) saudáveis de 18 agregados familiares foram rastreados para a presença de *K. pneumoniae* e *P. mirabilis* em amostras fecais.

A colonização intestinal por *K. pneumoniae* e *P. mirabilis* foi detetada em cães e humanos, contudo, nenhuma das estirpes isoladas era MDR.

As estirpes fecais de *K. pneumoniae* isoladas de cães pertenceram às linhagens clonais ST17, ST188, ST252, ST281, ST423, ST1093, ST1241, ST3398 e ST3399. Notavelmente, um dos agregados familiares (H15) incluiu dois cães que partilhavam uma estirpe cada com um dos humanos a viver no mesmo agregado familiar. As estirpes de *K. pneumoniae* partilhadas entre os pares cão/humano pertenciam ao mesmo ST (ST252 e ST1241) e apresentavam o mesmo padrão de restrição de PFGE (Dice/UPGMA, *Xba*I, 1,5% de tolerância), perfil de resistência aos antimicrobianos e genótipo de virulência. Foram detetados também dois humanos e um cão, de agregados familiares distintos, colonizados por uma estirpe de *K. pneumoniae* ST17 com perfil de restrição de PFGE idêntico e fenótipo de resistência aos antimicrobianos diferenciado.

No caso de *P. mirabilis,* os cães apresentaram uma frequência de colonização intestinal significativamente superior aos humanos (44,4% e 12,5%, respetivamente; *P* = 0,0329). Neste caso, um par cão/humano e um par cão/cão partilhavam estirpes de *P. mirabilis* com padrão de restrição de PFGE similar (Dice/UPGMA, *Not*I, 1,5% de tolerância).

Os resultados apresentados nesta tese, quanto à distribuição de resistência aos antimicrobianos em bactérias isoladas de animais de companhia com ITU na Europa e Portugal (Lisboa) , suportam a necessidade de implementação de programas de *antimicrobial stewardship*, sobretudo nos países do sul da Europa (Portugal, Espanha, Itália e Grécia). A deteção de bactérias MDR pertencentes a linhagens clonais de elevado risco nos animais de companhia com ITU e as semelhanças detetadas entre *K. pneumoniae* e *P. mirabilis* de origem animal e humana levantam preocupações de saúde pública e corroboram a necessidade de uma abordagem *One Health*.

Palavra chave: *Enterobacteriaceae*, ITU, animais de companhia, reservatório, epidemiologia.

Abstract

Uropathogenic *Proteus mirabilis* and *Klebsiella pneumoniae* in companion animals: molecular epidemiology, antimicrobial resistance and zoonotic potential

Urinary tract infections (UTI) are frequently diagnosed in companion animals and the increase in antimicrobial resistance leads to therapeutic limitations and public health concerns.

The study of the geographic distribution of antimicrobial resistance in bacteria (*n*= 22 256) causing UTI in companion animals from 14 European countries showed that, in 2012-2013, the frequency of *Escherichia coli* and *Proteus mirabilis* antimicrobial resistance in Southern countries (Portugal, Spain, Italy, Greece) was significantly higher than in Northern countries (Denmark, Sweden). In a retrospective study conducted in Portugal (Lisbon), the antimicrobial resistance of clinical Enterobacteriaceae from companion animals with UTI increased significantly over 16 years (1999-2014; $P < 0.001$). Bacteria from companion animals with UTI harboured important antimicrobial resistance mechanisms and belonged to high-risk clonal lineages, namely third-generation cephalosporin (3GC)-resistant *E. coli* O25b:H4-B2-ST131, CC23 and ST648, methicilin-resistant *Staphylococcus aureus* CC5, methicilin-resistant *Staphylococcus epidermidis* CC5, high-level gentamicin-resistant *Enterococcus faecalis* CC6 and ampicillin-resistant *Enterococcus faecium* CC17.

The $bla_{\text{CTX-M-15}}$ gene was disseminated among 3GC-resistant *K. pneumoniae* from companion animals and humans with UTI. Most *K. pneumoniae* from companion animals where 3GC, multidrug-resistant (MDR) and belonged to the high-risk clonal lineage ST15. *K. pneumoniae* high-risk clonal lineages ST11, ST37 and ST147 were also detected in companion animals.

3GC-resistance in *P. mirabilis* from companion animals with UTI was associated with the presence of $bla_{\text{CMY-2}}$, which increased significantly over time. A high number of PFGE clusters (43.6%, $n = 17/39$) included *P. mirabilis* strains from companion animals and humans.

Gut colonisation by *K. pneumoniae* and *P. mirabilis* was detected in healthy dogs and humans; however, none of the strains was MDR. *K. pneumoniae* faecal strains from dogs belonged to ST17, ST188, ST252, ST281, ST423, ST1093, ST1241, ST3398 and ST3399. Remarkably, two colonised dogs were found to shared PFGE undistinguishable *K. pneumoniae* strains with one co-living human. *P. mirabilis* gut colonisation was significantly higher in dogs ($P = 0.0329$). One human/dog and one dog/dog pair shared PFGE undistinguishable *P. mirabilis* strains.

The antimicrobial resistance frequencies reported in these studies support the need to implement antimicrobial stewardship programmes in veterinary medicine. The detection of MDR high-risk clonal lineages causing UTI in companion animals and the similarities detected in *K. pneumoniae* and *P. mirabilis* from companion animals and humans raises public health concerns and highlights the need for a One Health approach.

Keyword: *Enterobacteriaceae*, UTI, companion animals, reservoirs, epidemiology.

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Chapter 6 Discussion, conclusions and future perspectives

%	Per cent
16S rRNA	16S ribosomal RNA
16S rRNA methylases	16S ribosomal RNA methyltransferases
3GC	Third-generation cephalosporins
95% CI	Confidence interval of 95%
AAC	*N*-acetyltransferases
AK	Amikacin
AMC	Amoxicillin/clavulanate
AME	Aminoglycoside modifying enzymes
AMK	Amikacin
AMP	Ampicillin
AmpC	AmpC cephalosporinases
ANT	*O*-nucleotidyltransferases
APH	*O*-phosphotransferases
AT	Austria
ATCC	American type culture collection
ATF	Ambient-temperature fimbriae
ATM	Aztreonam
BE	Belgium
C	Chloramphenicol
CA-human	Human community-acquired UTI
CAT	Chloramphenicol acetyltransferase
CAUTI	Catheter associated UTI
CAZ	Ceftazidime
CC	Clonal complex
CEBEA	Comissão de Ética e Bem-Estar Animal
CEF	Cephalothin
CG	Clonal group
cgMLST	Core multilocus sequence typing
CH	Switzerland
CHL	Chloramphenicol
CI	Confidence interval of 95%
CIA	Critically important antimicrobial
CIP	Ciprofloxacin
CLSI	Clinical and laboratory standards institute
CN	Gentamicin

CoNS	Coagulase negative staphylococci
CoPS	Coagulase positive staphylococci
CPD	Cefpodoxime
CTX	Cefotaxime
CVN	Cefovecin
DE	Germany
DHFR	Enzyme dihydrofolate reductase
DHPS	Enzyme dihydropteroate synthase
DK	Denmark
DNA	Deoxyribonucleic acid
EARS-Net	European antimicrobial resistance surveillance network
EFT	Ceftiofur
EL	Greece
EL	Greece
EMA	European medicines agency
ENR	Enrofloxacin
EQUC	Enhanced quantitative urine culture
ERIC	Enterobacterial repetitive intergenic consensus sequence
ES	Spain
ESBL	Extended spectrum β-lactamases
ESKAPE	ESKAPE is an acronym encompassing the names of six bacterial pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, Acinetobacter baumannii, *Pseudomonas aeruginosa* and *Enterobacter*.
ETP	Ertapenem
EUCAST	European committee on antimicrobial susceptibility testing
FEP	Cefepime
FFC	Florfenicol
FLU	Fluoroquinolones
FOF	Fosfomycin
FOX	Cefoxitin
FQs	Fluoroquinolones
FR	France
FullS	Full-susceptibility
GEN	Gentamicin
h	Hour
HA-human	Human hospital patients with UTI
HestP	Highest priority

HIA	Highly important antimicrobial
HLGEN	High-level gentamicin
HLGR	High-level gentamicin-resistant
Human-CA	Human community-acquired UTI
Human-HA	Human hospital patients with UTI
HP	High priority
I	Intermediate
IA	Important antimicrobial
ICE	Integrative and conjugative elements
IMP	Imipenem
IQR	Interquartile range
IT	Italy
K-locus	*cps* locus
KAN	Kanamycin
LppA	Murein lipoprotein
LPS	Lipopolysaccharide
LVX	Levofloxacin
MALDI-TOF	Matrix assisted laser desorption ionization - time of flight mass spectrometry
MAR	Marbofloxacin
MCK	MacConkey agar plates
MDR	Multidrug-resistant
MEM	Meropenem
MET	Methicillin
MIC	Minimum inhibitory concentration
mL	Millilitre
MLST	Multilocus sequence typing
MR/K	Mannose-resistant *Klebsiella*-like fimbriae
MR/P	Mannose-resistant *Proteus*-like fimbriae
MRCoNS	Methicillin-resistant coagulase negative staphylococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
NaCl	Sodium chloride
NA	Nalidixic acid
NET	Netilmicin
NIT	Nitrofurantoin
NL	The Netherlands

NOR	Norfloxacin
ºC	Degree celsius
OMP	Outer membrane protein
OR	Odds ratio
OX	Oxacillin
P	Penicillin
PaI	Peptidoglycan-associated lipoprotein
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
PDR	Pandrug-resistant
PFGE	Pulse field gel electrophoreses
PGI1	*Proteus* genomic island 1
PMF	*Proteus mirabilis* fimbriae
PMP	*P. mirabilis* P-like fimbriae
PMQR	Plasmid-mediated quinolone resistance
PT	Portugal
Pta	*Proteus* toxic agglutinin
QRDR	Quinolone resistance-determining regions
R	Resistant or Fully-resistant according to the study
RADP	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rMLST	Ribosomal multilocus sequence typing
RS	Serbia
s	Seconds
S	Susceptible
SA	*Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome *mec*
SD	Standard deviation
SE	Sweden
SGI1	*Salmonella* genomic island 1
SNP	Single nucleotide polymorphisms
SP	*Staphylococcus pseudintermedius*
spa	Staphylococcal protein A
SPP	Other staphylococci
ST	Sequence type
Stat. Dif.	Statistical significant differences
SXT	Trimethoprim/sulfamethoxazole
TE	Tetracycline

TET	Tetracycline
TOB	Tobramycin
TZP	Piperacillin/tazobactam
UCA/NAF	Urothelial cell adhesion fimbriae/non-agglutinating fimbriae
UK	United Kingdom
UPEC	Uropathogenic *Escherichia coli*
UPGMA	Unweighted pair group method using arithmetic averages
USA	United States of America
UTI	Urinary tract infection
UTI-C	Community patient with UTI
UTI-H	Hospitalised patient with UTI
UTIR-VNet	Urinary tract infection resistance – veterinary network
V/cm^2	Volts per square centimetre
VetCAST	Veterinary subcommittee on antimicrobial susceptibility testing
VG	Virulence genotypes
wgMLST	Whole-genome multilocus sequence typing
WGS	Whole genome sequencing
XDR	Extensively drug-resistant
YHPI	Yersiniabactin high-pathogenicity island
μg	Microgram
μL	Microlitre

Chapter 1

Introduction

1.1 Urinary tract infections

Urinary tract infections (UTI) are frequently diagnosed in humans and companion animals (Harding & Ronald, 1994; Weese et al., 2011). In Humans from the United States, UTIs account for 0.7% of primary diagnosis at ambulatory care visits (Schappert & Rechtsteiner, 2011). When considering human healthcare-associated infections in the United States and Europe, the prevalence of UTIs is much higher (European Centre for Disease Prevention and Control [ECDC], 2013; Magill et al., 2014). Ling[1] (Ling 1984 cited by Thompson, Litster, Platell & Trott, 2011) has estimated that around 14% of dogs visiting a veterinarian will develop a UTI during their life time. The frequency of urinary tract infections among cats with lower urinary tract disease has been considered to be low (<2%) (Kruger et al., 1991; Buffington et al., 1997; Gunn-Moore, 2003); however, some studies found higher frequencies, varying between 8% and 25% (Lekcharoensuk, Osborne & Lulich, 2001; Gerber et al., 2005; Eggertsdóttir, Lund, Krontveit & Sørum, 2007; Sævik, Trangerud, Ottesen, Sørum & Eggertsdóttir, 2011).

1.1.1 Pathogenesis of bacterial urinary tract infections

UTI is a consequence of the host defence mechanisms failure allowing the adherence, multiplication and persistence of virulent bacteria in the urinary tract (Bartges, 2004).

UTI usually initiates with the adherence and colonisation of bacteria into the urethra followed by migration to the bladder. Complex host-pathogen interactions will determine the outcome of the bacteria migration. When successful, the bacteria will multiply and colonise the bladder and may ascend to the kidney. Ultimately, bacteria will cross the tubular epithelial barrier into the blood stream, resulting in bacteraemia (Flores-Mireles, Walker, Caparon & Hultgren, 2015).

Ascending bacteria from the lower urinary tract is the main route of upper urinary tract infection as opposed to haematogenous/lymphatic route or infection from surrounding tissues (Thompson, 2011; Smee, Loyd & Grauer, 2013). Most of the bacteria causing UTI originate from the gastrointestinal tract or skin surrounding the vulva and prepuce (Bartges, 2004).

[1] Ling, G.V. (1984). Therapeutic strategies involving antimicrobial therapy of the canine urinary tract. *Journal of the American Veterinary Medical Association*, 185, 1162–1164.

The adherence, migration and persistence of bacteria in the urinary tract relies on several virulence factors such as: fimbriae (pili) and adhesins that recognise receptors on the urinary tract epithelium and mediate colonisation/migration; toxins and proteases that release nutrient from host cells; and siderophores that mediate iron acquisition (Flores-Mireles et al., 2015).
Host predisposing diseases associated with higher UTI frequencies include *diabetes mellitus* in dogs, cats and humans; chronic kidney disease in cats and humans; hyperthyroidism in cats and hyperadrenocorticism and bladder transitional cell carcinoma in dogs (Saitoh, Nakamura, Hida & Satoh, 1985; Forrester, Troy, Dalton, Huffman & Holtzman, 1999; Hess, Saunders, Van Winkle & Ward, 2000; Mayer-Roenne, Goldstein & Erb, 2007; Bailiff et al., 2006; Hirji, Guo, Andersson, Hammar & Gomez-Caminero, 2012; Budreckis et al., 2015). Seguin et al. (2003) found that dogs with urine specific gravity higher than 1.025 are less likely to develop UTI by *E. coli*. Martinez-Ruzafa et al. (2012) detected a significant association between low urine specific gravity and UTI in cats. Contrary, several studies found that dogs and cats with low urine specific gravity do not seem to be predisposed to UTI (McGuire, Schulman, Ridgway & Bollero, 2002; Seguin, Vaden, Altier, Stone & Levine, 2003; Bailiff et al., 2008). The existence of anatomical abnormalities or diseases promoting urine retention and abnormal micturition (e.g. urinary obstruction or neurologic diseases) also promote UTI (Seguin et al., 2003; Bubenik & Hosgoof, 2008; Stiffler et al., 2006; Graves et al., 2007; Eriksson, Gustafson, Fagerström & Olofsson, 2010; Martinez-Ruzafa et al., 2012). Furthermore, corticoid treatment also seems to predispose to UTI (Freshman, Reif, Allen & Jones, 1989; Torres et al., 2005).
UTI is more frequent in female dogs, cats and humans (Lekcharoensuk et al., 2001; Ling et al., 2001; Cohn, Gary, Fales & Madsen, 2003; Bailiff et al., 2006; Graves et al., 2007; Mayer-Roenne et al., 2007; Bailiff et al., 2008; Flores-Mireles et al., 2015). The mean age of UTI diagnosis is similar between female and male dogs ranging between 7 and 8 years-old (Ling et al., 2001; Seguin et al., 2003; Cohn et al., 2003); nevertheless, age may vary widely (Norris et al., 2000). Spayed female dogs have higher risk for developing UTI (Cohn et al., 2003; Seguin et al., 2003). Persian and Abyssinian breeds, increasing age and low body weight are associated with increased risk of UTI in cats (Lekcharoensuk et al., 2001; Bailiff et al., 2008). In men, the risk of UTI increases with age (Griebling, 2005). Other, human related predisposing factors for UTI include intercourse frequency/partner, use of certain contraceptives, hygiene patterns, indwelling urinary catheters, prior UTI, among others (Foxman et al., 2000; Scholes et al., 2000; Graves et al., 2007; Badran et al., 2015).

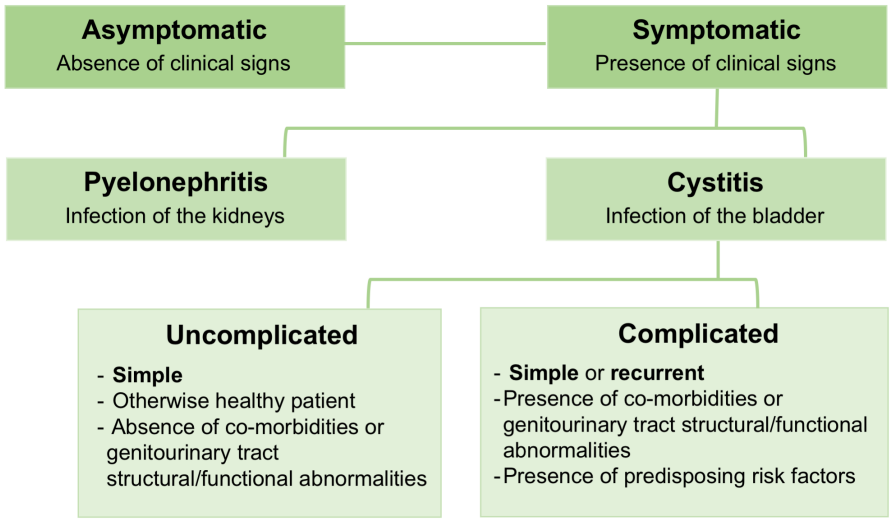
1.1.2 Classification of urinary tract infections

In veterinary medicine, there is a dedicated guideline for the antimicrobial treatment of UTI in companion animals that was developed by a Working Group of the International Society for Companion Animal Infectious Diseases (Weese et al., 2011). One focal point of the ITU treatment decision making in this guideline relies on the classification of the type of UTI.

In human medicine, there are several UTI guidelines using different classification systems (European Association of Urology [EAU], 2017). Johasen et al. (2011) revised the main human guidelines used at the time and proposed a new classification system accounting for the clinical presentation of the UTI, categorisation of UTI risk factors and availability of appropriate antimicrobial therapy. Recently, this guideline was further improved (Smelov, Naber & Johansen, 2016; EAU, 2017).

The human UTI classification system (Smelov et al., 2016) is more complex and the main classification groups vary from the ones proposed by Weese et al. (2011) for companion animals (Figure 1 and Figure 2).

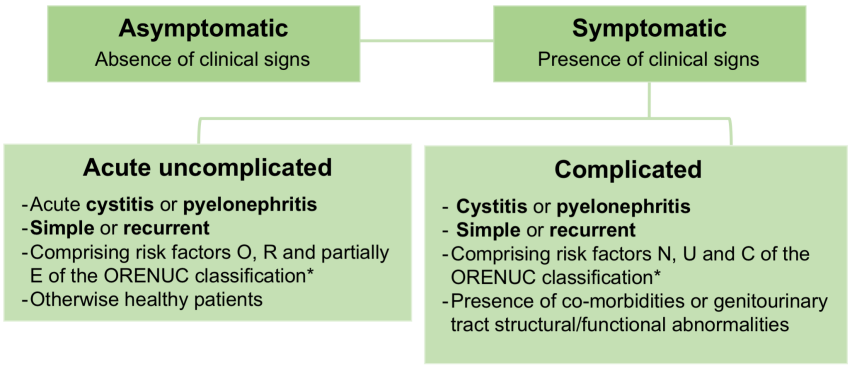
Figure 1 – Classification of UTIs in companion animals according to Weese et al. (2011)



Bacteriuria can be detected in patients with or without clinical signs (symptomatic *versus* asymptomatic) (Weese et al., 2011; Smelov et al., 2016). Clinical signs are not pathognomonic and may include dysuria, pollakiuria, stranguria, haematuria, urgency to urinate, fever, abdominal/flank pain, vocalisation (in companion animals), among others (Bartges et al., 2004; Gerber et al., 2005; Weese et al., 2011; Johasen et al., 2011; Passmore, Sherington & Stegemann, 2008).

According to the clinical presentation of symptomatic bacteriuria, UTIs are considered upper UTIs or pyelonephritis (kidney) or lower UTIs or cystitis (bladder) (Weese et al., 2011; Smelov et al., 2016). Human UTI guidelines also consider urosepsis as an additional clinical presentation and grade clinical signs in 6 levels of severity: cystitis, mild and moderate pyelonephritis, severe pyelonephritis, urosepsis, severe urosepsis and urosepsic shock (Smelov et al., 2016).

Figure 2 - Classification of UTIs in humans according to Smelov et al. (2016)



Legend: *please refer to Table 2 to obtain information about the ORENUC classification system.

Based on the frequency of UTI episodes within the last 12 months, UTIs can be classified as simple (<3 episodes) or recurrent (≥3 episodes) (Weese et al., 2011; Smelov et al., 2016). Repeated UTIs may be considered as reinfection, relapse or refractory UTIs (Table 1).

Table 1 – Repeated UTIs

	Description
Reinfection*	- within 6 months of cessation of previous UTI, - after apparent successful treatment, - isolation of a different microorganism.
Relapse*	- within 6 months of cessation of previous UTI (usually weeks), - after apparent successful treatment, - isolation of an indistinguishable organism from the previous UTI.
Refractory	- persistently positive culture during treatment, - no period of negative bacteriuria during or after treatment, - isolation of an indistinguishable organism from the previous UTI.

Legend: *subtype of recurrent UTI

Uncomplicated UTIs in companion animals, include cystitis diagnosed in patients that are otherwise healthy (i.e. without comorbidities) and with normal genitourinary tract anatomy and function (Weese et al., 2011). Uncomplicated UTIs in companion animals are always simple UTIs, since recurrent UTIs are considered by Weese et al. (2001) as a sign of presence of undiagnosed comorbidities. The uncomplicated UTI definition for humans differs significantly, since besides simple acute cystitis it also includes recurrent acute cystitis and simple and recurrent acute pyelonephritis (Smelov et al., 2016). Furthermore, in human guidelines, risk factors for UTI are graded according to the ORENUC classification, and patients with low severity risk factors are included in the uncomplicated UTI group (Table 2) (Smelov et al., 2016).

Complicated UTI in companion animals are those occurring in patients with comorbidities (e.g. urinary obstruction, renal failure, *diabetes mellitus*) or predisposing factors for UTI such as anatomic or functional abnormalities of the genitourinary tract (Weese et al., 2011). As

mentioned above, all recurrent UTIs are considered as complicated UTIs in companion animals (Weese et al., 2011). In humans, a complicated UTI is a simple/recurrent cystitis/pyelonephritis associated with a structural or functional abnormality of the genitourinary tract or an underlying disease with high risk for severe outcome (Figure 2 and Table 2) (Smelov et al., 2016). Finally, in companion animals, pyelonephritis is considered a UTI group by itself (Weese et al., 2011).

Table 2 – Host risk factors in UTI categorised according to the ORENUC system from the human UTI guidelines (adapted from Smelov et al., 2016)

Phenotype	Category of risk factor
O	No known risk factor.
R	Risk factors for recurrent urinary tract infection but no risk of more severe outcome.
E	Extraurogenital risk factors with risk of more severe outcome.
N	Nephropathic diseases with risk of more severe outcome.
U	Urologic risk factors with risk of more severe outcome, which can be resolved during therapy.
C	Permanent urinary catheter and unresolvable urologic risk factors with risk of more severe outcome.

Since, clinical signs of UTI are non-specific, UTI diagnosis and classification should include the clinical evaluation of the patient, complete urinalysis including urine specific gravity and sediment analysis, the necessary diagnostic workout to detect suspected comorbidities, and quantitative urine culture (Wilson & Gaido, 2004; Weese et al., 2011). The presence of bacteriuria and pyuria (more than three to five white blood cells per high-power field) in urine sediment strongly correlates with the presence of UTI, however it is not diagnostic (Kahlmeter & ECO.SENS, 2003; Bartges et al., 2004; Mayer-Roenne, 2007). Quantitative urine culture is the gold standard for diagnosis of significant bacteriuria[2] (Bartges et al., 2004; Weese et al., 2011; Smelov et al., 2016) and ideally should always be performed together with antimicrobial susceptibility testing to confirm empiric diagnosis, to adjust initial empiric therapeutics, to guide in recurrent UTI diagnosis and to gather epidemiological data on local aetiology and susceptibility patterns (Weese et al., 2011).

In companion animals, urine should be preferably collected by cystocentesis, followed by catheterisation or free-catch (midstream voiding or manual expression) to avoid 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). In humans, midstream voiding is frequently used because it is less invasive and less time consuming than

[2] Significant bacteriuria – this term is used to describe bacteriuria associated with UTI, and when in the absence of clinical signs, asymptomatic bacteriuria (Bartges et al., 2004).

cystocentesis and catheterisation (Wilson & Gaido, 2004). Urine culture should always be quantitative so that significant bacteriuria can be diagnosed accounting for the number of colony forming units per urine volume and the urine collection method (Bartges et al., 2004; Wilson & Gaido, 2004; Smelov et al., 2016).

1.1.3 Aetiology

Until recently, the bladder was believed to be a sterile environment, however due to the arising of high-throughput DNA-sequencing techniques, the urine microbiota started to be unravelled in healthy humans and dogs (Wolfe et al., 2012; Burton et al., 2017). Results from several studies show that part of the urine "healthy microbiota" is composed of slow-growing bacteria, therefore not detectable using classic aerobic urine culture procedures (Brubaker & Wolfe, 2017; Hilt et al., 2014). These findings lead Hilt et al. (2014) to propose an enhanced quantitative urine culture (EQUC) procedure that succeeded in demonstrating the existence of live microbiota in urine. Nevertheless, the role of the "healthy microbiota" and slow growing or anaerobic micro-organisms in urine from healthy individuals still needs to be explored (Brubaker & Wolfe, 2017). Interestingly, early studies on asymptomatic bacteriuria in women have suggested that it might have a protective effect against UTI (Cai et al., 2012).

Despite the new developments on the urine microbiota, current knowledge of UTI aetiology is still mainly based on quantitative urine culture conducted by classic microbiology.

Studies on UTI from companion animals report different proportions of aetiological agents maybe reflecting different geographical/temporal trends as well as different inclusion criteria. Geographical differences in the aetiology of UTIs has been previously pointed out in humans (Kahlmeter & ECO.SENS, 2003). The different inclusion criteria among studies and the different UTI classification in human and veterinary medicine limits straightforward comparisons among and between companion animals and humans.

Usually UTIs are caused by a single bacterium, but polimicrobial infections may also occur (Hess et al., 2000; Ling et al., 2001; Litster, Moss, Honnery, Rees & Trott, 2007b; Forrester et al., 1999). In humans, dogs and cats, *E. coli* (uropathogenic *E. coli* - UPEC) is by far the most common isolated bacteria in all types of UTIs. Nonetheless, UTIs can be caused by other Gram-negative and Gram-positive bacteria, and more rarely by fungi and viruses (Bush, 1976; Wooley & Blue, 1976; Forrester et al., 1999; Gupta, Hooton, Wobbe & Stamm, 1999; Hess et al., 2000; Ling et al., 2001; Cohn et al., 2003; Kahlmeter & ECO.SENS, 2003; Pressler, Vaden, Lane, Cowgill & Dye, 2003; Torres et al., 2005; Bailiff et al., 2006; Litster et al., 2007b; Mayer-Roenne et al., 2007; Passmore et al., 2008; Martinez-Ruzafa et al., 2012; Dorsch, von Vopelius-Feldt, Wolf, Straubinger & Hartmann, 2015; Flores-Mireles et al., 2015; Moyaert et al., 2017).

Cats are usually reported as having a high frequency of UTIs caused by *Enterococcus* spp. and *Staphylococcus* spp. (Wooley & Blue, 1976; Bailiff et al., 2006; Litster et al., 2007b; Mayer-

Roenne et al., 2007; Bailiff et al., 2008; Passmore et al., 2008; Martinez-Ruzafa et al., 2012; Dorsch et al., 2015; Moyaert et al., 2017; Teichmann-Knorrn, Reese, Wolf, Hartmann & Dorsch, 2018). *Enterococcus faecalis* is the most prevalent *Enterococcus* species isolated in cats with UTI, followed by *Enterococcus faecium* that is rarely isolated (Litster et al., 2007b; Mayer-Roenne et al., 2007). Several *Staphylococcus* species may cause UTI in cats including *Staphylococcus aureus* and *Staphylococcus pseudintermedius* (Litster et al., 2007b; Couto et al., 2016). Recently, Litster et al. (2007b) highlighted the high frequency of UTIs caused by *Staphylococcus felis* in cats from Australia. Other less frequent bacteria causing UTIs in cats include *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Pasteurela* spp., *Klebsiella pneumoniae*, among others. Frequencies of UTI aetiological agents vary according to the study design (Wooley & Blue, 1976; Lekcharoensuk et al., 2001; Bailiff et al., 2006; Bailiff et al., 2008; Litster et al., 2007b).

In dogs, *P. mirabilis* usually is the second most common Enterobacteriaceae causing UTI (Wooley & Blue, 1976; Ling et al., 2001; Cohn et al., 2003; Moyaert et al., 2017). Other common causes of UTI in dogs include *Staphylococcus* spp., *Enterococcus* spp. and more rarely *Pseudomonas* spp., *Klebsiella* spp., *Streptococcus* spp., among others (Forrester et al., 1999; Norris et al., 2000; Ling et al., 2001; Prescott, Hanna, Reid-Smith & Drost, 2002; Cohn et al., 2003). *Staphylococcus pseudintermedius* is the most common *Staphylococcus* species isolated from dogs with UTI, although several other species have been identified (Prescott et al., 2002; Cohn et al., 2003; Penna, Varges, Martins, Martins & Lilenbaum, 2010). Studies in dogs with complicated/recurrent UTI show 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 identified different bacteria frequencies according to the dog gender (Norris et al., 2000; Ling et al., 2001; Cohn et al., 2003).

K. pneumoniae and *P. mirabilis* are the most common Gram-negative bacteria in women with uncomplicated UTI after *E. coli* (Gupta et al., 1999). Gram-positive bacteria are frequently isolated, namely *Staphylococcus saprophyticus* and less frequently *Enterococcus* spp. (Gupta et al., 1999; Kahlmeter & ECO.SENS, 2003). *S. saprophyticus* has also been found to cause UTIs in dogs (Penna et al., 2002). When considering complicated and health-care associated UTIs, *Klebsiella* spp., *Enterococcus* spp., and other less common bacteria occur more frequently (Ronald, 2003; Wagenlehner, Niemetz, Weidner & Naber, 2007; Magill et al., 2014; Flores-Mireles et al., 2015).

1.2 Antimicrobial treatment for UTI and antimicrobial resistance genes

The increasing antimicrobial resistance trends in humans and companion animals are a worldwide concern (World Health Organization [WHO], 2017a). The selective pressure employed due to antimicrobial use is believed to be a key factor contributing to the rising

antimicrobial resistance trends (Guardabassi, Schwarz & Lloyd, 2004; Paterson & Bonomo, 2005; Pomba et al., 2017; WHO, 2017a). Since antimicrobials are the cornerstone of UTI treatment and are frequently required, correct UTI diagnosis and proper antimicrobial selection is crucial to avoid antimicrobial misuse (Weese et al., 2011). Human and companion animal guidelines for UTI treatment propose a rational list of antimicrobials to be used according to the type of UTI (Weese et al., 2011; EAU, 2017) (Table 3). These guidelines should be adjusted considering the geographic particularities such as antimicrobial resistance rates, antimicrobial availability and prescribing regulations (Weese et al., 2011; EAU, 2017).

Generally, antimicrobial treatment in asymptomatic bacteriuria it is not required (Weese et al., 2011; EAU, 2017); however, treatment may be considered in companion animals with high risk of ascending/systemic infection (Weese et al., 2011).

Antimicrobials for UTI treatment ideally should achieve high concentrations in the active form in urine. The main antimicrobials used for UTI treatment in veterinary medicine are also used in human medicine, namely β-lactams, fluoroquinolones and trimethoprim/sulfamethoxazole (Table 3). The WHO (2017a) has grouped antimicrobials according to their importance to human medicine into 3 categories (important, highly important and critically important antimicrobials [CIA]) using two criteria (Annex 1). CIA were recently further divided into high or highest priority antimicrobials based on 3 additional prioritisation criteria (Annex 1) (WHO, 2017a). It is important to stand out that most antimicrobials approved for UTI treatment in veterinary medicine are considered CIAs of highest priority (third-generation cephalosporins and fluoroquinolones) and CIAs of high priority (aminoglycosides, aminopenicillins) (Table 3). Therefore, careful choice of the antimicrobial therapy in veterinary medicine is of the upmost importance. The choice of the appropriate antimicrobial should always be supported on culture and antimicrobial susceptibility data (Weese et al., 2011). However, empirical antimicrobial treatment is sometimes necessary to relieve patient discomfort and prevent systemic infection (Weese et al., 2011). Sørensen et al. (2018) showed that a high percentage of dogs with suspected UTI are unnecessarily treated empirically regardless of the diagnostic work up followed. Interistingly, the EAU (2017) accepts that human UTI diagnosis of uncomplicated cystitis may be reasonably based on urine dipstick testing alone together with a complete clinical evaluation. When needed, the empirical antimicrobial choice should rely on first-line antimicrobials and then adjusted based on antimicrobial susceptibility data if necessary (Weese et al., 2011). Updated epidemiological data (aetiology and resistance trends) is crucial in guiding empirical treatment since bacteria may have specific intrinsic[3] (Table 4) and acquired resistance mechanisms. According to the Infectious Diseases Society of America guidelines for treatment of acute uncomplicated cystitis and pyelonephritis in women, an antimicrobial

[3] "Intrinsic (inherent) resistance, as opposed to acquired and/or mutational resistance, is a characteristic of all or almost all isolates of the bacterial species. The antimicrobial activity of the drug is clinically insufficient or antimicrobial resistance is innate, rendering it clinically useless." (Leclercq et al., 2013).

Table 3 – Recommended antimicrobials for UTI treatment in companion animals and humans

Antimicrobials	WHO (2017a) classification[1]	Companion animals[2]	Humans[3]
β-Lactams			
Amoxicillin	CIA - HP	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI.	Not recommended for empiric treatment of uncomplicated cystitis or complicated UTI due to high resistance rates. May be used in selected cases. Recommended for complicated UTI treatment in combination with aminoglycosides.
Amoxicillin/clavulanate	CIA - HP	Unknown advantage over amoxicillin alone. If amoxicillin-resistance rates are high, it may be a good first-line option for the treatment and empirical treatment of uncomplicated and complicated UTI.	Not recommended for empiric treatment of uncomplicated cystitis or complicated UTI due to high resistance rates. May be used in selected cases.
Second and third-generation cefalosporins	CIA – HestP (third-generation) HIA (second-generation)	Second-line option for UTI treatment.	Oral cephalosporins are not recommended for empiric treatment of uncomplicated cystitis due to high resistance rates. May be used in selected cases. Third-generation cephalosporins are recommended for uncomplicated pyelonephritis treatment. Recommended for complicated UTI treatment in combination with aminoglycosides.
Carbapenems	CIA - HP	Last-resort antimicrobial. Off-lable use.[4]	Last-resort antimicrobial. Suitable for uncomplicated pyelonephritis treatment.
Other β-lactams	HP (pivmecillinam, cefadroxil) CIA - HP (piperacillin/tazobactam) CIA - HestP (cefepime)	-	Pivmecillinam: First-line option for uncomplicated cystitis. Not recommended for pyelonephritis treatment. Cefadroxil: Second-line option for uncomplicated cystitis. Piperacillin/tazobactam, cefepime: Suitable for complicated UTI treatment.

Table 3 (continuation) - Recommended antimicrobials for UTI treatment in companion animals and humans

Antimicrobials	WHO (2017a) classification[1]	Companion animals[2]	Humans[3]
Aminoglycosides	CIA - HP	Not recommended for routine use due to side effects. Although not included in Weese et al. (2011) guidelines, gentamicin is suitable for UTI treatment (Ling & Ruby, 1979).	Suitable for uncomplicated pyelonephritis treatment. Recommended for complicated UTI in combination with amoxicillin or third-generation cephalosporins.
Chloramphenicol	HIA	Off-lable use.[4] Recommended for multidrug-resistant bacteria.	-
Doxycycline	HIA	Not recommended for routine use.	May be used for treatment of uncomplicated cystitis in patient with renal insufficiency.
Fluoroquinolones	CIA - HestP	Second-line option for UTI treatment. Considered a good first-line option for empiric antimicrobial treatment of pyelonephritis.	Not considered first-line option for the treatment of uncomplicated cystitis due to ecological effects and selection for resistance. Recommended for uncomplicated pyelonephritis treatment. May not be suitable for empiric treatment of complicated UTIs.
Fosfomycin	CIA - HP	Not included Weese et al. (2011) guidelines.	First-line option for empirical treatment of uncomplicated cystitis. Not recommended for pyelonephritis treatment.
Nitrofurantoin	IA	Second-line antimicrobial. Off-lable use.[4] Reserved for uncomplicated UTIs caused by multidrug-resistant bacteria.	First-line option for empirical treatment of uncomplicated cystitis. Not recommended for pyelonephritis treatment.
Trimethoprim/sulfamethoxazole	HIA	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI.	Trimethoprim or trimethoprim/sulfamethoxazole is a second-line option for uncomplicated cystitis. Can be considered a first-line option if local *E. coli* resistance rates are lower than 20%. May be suitable for uncomplicated pyelonephritis treatment. Not recommended for empiric treatment of complicated UTI due to high resistance rates.

Legend:[1]CIA, critically important antimicrobial; HIA, highly important antimicrobial; IA, important antimicrobial; HP, high priority; HestP, highest priority; [2]Major data according to Weese et al. (2011); [3]Data according to EAU (2017); [4]Off-label use is defined in Directive 2001/82/EC as 'the use of a veterinary medicinal product that is not in accordance with the summary of the product characteristics, including the misuse and serious abuse of the product' (European Medicines Agency [EMA], 2018).

Table 4 – Intrinsic resistances of common bacteria causing UTI (European Committee on Antimicrobial Susceptibility Testing, 2016)

Bacteria	Intrinsic resistance
Enterobacteriaceae	Benzylpenicillin, glycopeptides, fusidic acid, macrolides (with some exceptions), lincosamides, streptogramins, rifampicin, daptomycin and linezolid.
Escherichia coli	-
Klebsiella pneumoniae	Ampicillin and ticarcillin.
Proteus mirabilis	Nitrofurantoin, tetracycline, tigecycline, polymixin B and colistin.
Non-fermentative Gram-negative	Benzylpenicillin, first- and second-generation cephalosporins, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin and linezolid.
Pseudomonas aeruginosa	Ampicillin, amoxicillin/clavulanate, ampicillin/sulbactam, cefazolin, cefalothin, cefalexin, cefadroxil, cefotaxime, ceftriaxone, ertapenem, chloramphenicol, kanamycin, neomycin, trimethoprim, tetracycline and tygecicline.
Gram-positive	Aztreonam, temocillin, polymyxin B/colistin and nalidixic acid.
Staphylococcus spp.	Ceftazidime, fusidic acid (*S. saprophyticus*), fosfomycin (*S. saprophyticus* and *S. capitis*) and novobiocin (*S. saprophyticus*, *S. cohnii* and *S. xylosus*).
Streptococcus spp.	Ceftazidime, fusidic acid and low-level amynoglicosides.
Enterococcus faecalis	Cephalosporins, fusidic acid, low-level amynoglicosides, erythromycin, clindamycin, quinupristindalfopristin and sulfonamides.
Enterococcus faecium	Cephalosporins, fusidic acid, low-level amynoglicosides[1], erythromycin and sulfonamides.

Legend: [1]produces a chromosomal AAC(6')-I enzyme leading to the loss of synergism between aminoglycosides (except gentamicin, amikacin and streptomycin) and penicillins or glycopeptides.

should only be considered suitable for empirical treatment of uncomplicated UTIs or upper UTIs if antimicrobial resistance frequencies are lower than 20% or 10%, respectively (Gupta et al., 2011).

It is interesting to notice that the recommended UTI treatment duration in companion animals is significantly longer than in humans (uncomplicated UTI: 3-5 days in humans, 7-14 days in companion animals; complicated UTI: 7-14 days in humans, 4 weeks in companion animals, 4-6 weeks in companion animals with pyelonephritis) (Weese et al., 2011; Smelov et al., 2016). In fact, treatment duration recommendations in companion animals are supported by little scientific evidence (Weese et al., 2011).

Few studies have reported short-duration antimicrobial treatment protocols for UTI in companion animals (Westropp et al., 2012; Clare et al., 2014). However, studies comparing the same antimicrobial regime with differing durations are lacking (Jessen, Sørensen, Bjornvad, Nielsen & Guardabassi, 2015).

1.2.1 β-Lactams

β-Lactams are among the most used antimicrobials in human and veterinary medicine (European Centre for Disease Prevention and Control [ECDC], 2016a; European Medicines Agency [EMA], 2017b), and are characterised by the presence of a β-lactam ring (Smet et al., 2010). β-lactams are bactericidal agents that prevent bacterial cell-wall synthesis by binding to an active serine site from penicillin-binding proteins (PBPs) in Gram-negative and Gram-positive bacteria (Smet et al., 2010; Bush & Bradford, 2016).

The first β-lactam (penicillin) was discovered by Alexander Fleming in 1929 (Fleming, 1929). Since then, several other β-lactams were discovered that may be grouped into penicillins, cephalosporins, carbapenems and monobactams (Bush & Bradford, 2016).

Penicillins include penicillin G and penicillin V, two natural occurring penicillins, initially and still used to treat streptococcal infections (Smet et al., 2010). The arising of resistant bacteria due to the production of β-lactamases prompted the discovery of new penicillins such as methicillin, oxacillin, cloxacillin, and nafcillin (used for staphylococcal infections) (Bush & Bradford, 2016). In the 1970s, ampicillin and amoxicillin with improved activity against Enterobacteriaceae were introduced (Bush & Bradford, 2016). This group also includes anti-pseudomonal penicilins (e.g. carbenicillin, piperacillin, ticarcillin), mecillinam and temocillin (Bush & Bradford, 2016). Amoxicillin is excreted in urine predominantly in the active form (Weese et al., 2011) and therefore is the recommended aminopenicillin for companion animal UTI treatment (Table 3).

The first cephalosporins were discovered in the 1950s (Asbel & Levison, 2000; Fernandes, Amador & Prudêncio, 2013). The high number of cephalosporins discovered to date, are grouped into 5 generations according to their spectrum of activity (Fernandes et al., 2013). Cephalosporin activity varies from Gram-positive to Gram-negative or both (Asbel & Levison, 2000; Fernandes et al., 2013). In veterinary medicine, the approved cephalosporins belong to the first to forth-generations (Smet et al., 2010). It is noteworthy that the third-generation cephalosporin available for UTI treatment in companion animal use (cefovecin) (Passmore et al., 2008) is a long action antimicrobial leading to higher impact on the selection of resistant bacteria.

Carbapenems, such as imipenem and meropenem, are last resort antimicrobials (critically important antimicrobial of highest priority) with resistance to most β-lactamases and activity against Gram-positive and Gram-negative bacteria (Papp-Wallace, Endimiani, Taracila & Bonomo, 2011; Fernandes et al., 2013; Bush & Bradford, 2016). These are not approved for veterinary use.

Finally, monobactams (aztreonam) have antimicrobial activity especially directed towards Gram-negative bacteria (Asbel & Levison, 2000), and are also not approved for veterinary use. Aztreonam use in UTI treatment in humans has been reported (Martelli, Cortecchia & Ventriglia, 1989).

β-Lactam-resistance can be caused by several mechanisms such as porin deficiency, overexpression of efflux pumps, modification of the PBPs and production of β-lactamases (Bush & Jacoby, 2010; Fernandes et al., 2013).

PBP mutations are responsible for an important resistance mechanism in *Staphylococcus* species, namely methicillin-resistance (Fernandes et al., 2013). The methicillin-resistant staphylococcus aureus (MRSA) is a major pathogen to humans (Hiramatsu, Cui, Kuroda & Ito, 2001). Methicillin-resistance is conferred by a mobile genetic element (staphylococcal chromosome cassette *mec* [SCC*mec*]) which contains the *mecA* gene that encodes a modified, but active, PBP (PBP2a) with lower affinity to penicillins, cephalosporins and carbapenems (Hiramatsu et al., 2001; Weese & van Duijkeren, 2010; Fernandes et al., 2013). More recently a *mecC* gene has been identified (García-Álvarez et al., 2011). Methicillin-resistant *Staphylococcus*, including MRSA, have been reported in companion animals (Weese, 2010; Weese & van Duijkeren, 2010; Couto et al., 2016).

The production of β-lactamases is the most common β-lactam-resistance mechanism in clinically relevant Gram-negative bacteria (Bush & Jacoby, 2010). Several β-lactamases are plasmid-mediated which leads to rapid worldwide resistance dissemination (Cantón & Coque, 2006; Fernandes et al., 2013).

β-Lactamase inhibitors were discovered in the attempt to overcome the β-lactamase activity of β-lactam-resistant bacteria (Bush & Bradford, 2016). Clavulanic acid acts synergistically with penicillins and cephalosporins allowing the β-lactam to kill bacteria producing clavulanic acid sensitive β-lactamases (Fernandes et al., 2013; Bush & Bradford, 2016). Other β-lactamase inhibitors include sulbactam and tazobactam (Bush & Bradford, 2016). In veterinary medicine the most used combination is amoxicillin/clavulanate.

The first evidence of β-lactamase activity was described in a *E. coli* by Abraham and Chain (1940) in 1940. The first Gram-negative plasmid-mediated β-lactamase, TEM-1, was detected in a *E. coli* in the 1960s (Fernandes et al., 2013). Since then, more than 1300 distinct β-lactamases have been characterised (Bush, 2013).

Through the years, several classification systems have been proposed aiming to systematise the big diversity of β-lactamase enzymes (Bush, 2013). Currently, the main classification system results from the fusion of the molecular classification initiated by Ambler (1980) and the Bush (1989) functional classification. This fusion was proposed by Bush, Jacoby and Medeiros (1995) and later updated by Bush and Jacoby (2010) (Table 5). Based on the molecular characterisation, β-lactamases may be divided in four main classes according to their amino acid sequence (A, B, C e D) (Bush, 2013). The functional classification is based on key β-lactam substrates and inhibitors which define 3 main β-lactamase groups and respective subgroups according to their hydrolytic and inhibitor profile (Bush, 2013) (Table 5).

Molecular class B (functional group 3a and 3b) β-lactamases are metallo-β-lactamases[4] while the remaining enzymes are serino-β-lactamases[5] (Bush, 2013; Bush & Jacoby, 2010).

Third-generation cephalosporins and carbapenems are CIAs of highest priority to humans (WHO, 2017a). Therefore, β-lactamase production has an important clinical impact, especially in healthcare settings (Bush, 2013; Fernandes et al., 2013). In fact, the World Health Organization has recognised third-generation cephalosporin and carbapenem-resistant Enterobacteriaceae as a highest priority pathogen for research (World Health Organization [WHO], 2017b). Third-generation cephalosporin and carbapenem hydrolysing β-lactamases are frequently plasmid-mediated leading, as mentioned above, to their easy dissemination (Cantón & Coque, 2006; Bush & Jacoby, 2010). Furthermore, β-lactamase plasmid-mediated resistance is associated with multidrug resistance due to co-transfer of several resistance mechanisms in the same plasmid which creates big therapeutic challenges (Cantón & Coque, 2006).

The commonly named extended-spectrum beta-lactamases (ESBL) are enzymes belonging to the 2be group (Bush & Jacoby, 2010), that hydrolyse penicillins, first, second and third-generation cefalosporins and aztreonam, and that are inhibited by clavulanic acid (Paterson & Bonomo, 2005). Although ESBLs are inhibited by clavulanic acid, due to limited reseach data, β-lactam/ β-lactam inhibitor combinations are not considered as suitable first line options for the treatment of serious infections caused by ESBL-producing bacteria (Paterson & Bonomo, 2005). Nevertheless, successful treatment using amoxicillin/clavulanate in humans with UTI caused by fully amoxicillin/clavulanate-susceptible ESBL-producing *E. coli* has been reported (Lagacé-Wiens et al., 2006; Beytur, Yakupogullari, Oguz, Otlu & Kaysadu, 2015).

ESBLs include clinically important β-lactamases from several enzyme families such as TEM, SHV and CTX-M (Bush & Jacoby, 2010). The first ESBLs were described in Europe in the 1980s, and since then ESBLs have been reported worldwide (Paterson & Bonomo, 2005; Fernandes et al., 2013). The CTX-M family has become the most frequent ESBL in bacteria causing health-care and community associated infections and is now endemic worldwide (Cantón & Coque, 2006; Doi, Iovleva & Bonomo, 2017), which reveals their public health relevance (Fernandes et al., 2013). There is host and geographic variation of the CTX-M enzymes distribution; nevertheless, some enzymes, such as CTX-M-15, are worldwide disseminated (Cantón & Coque, 2006; Nicolas-Chanoine et al., 2008; Smet et al., 2010; Ewers, Bethe, Semmler, Guenther & Wieler, 2012; Bevan, Jones & Hawkey, 2017). CTX-M-15 seems to be more common in humans, but it is also frequently found in companion animals (Coque, Baquero & Canton, 2008; Ewers et al., 2012). CTX-M-1 is the most frequent CTX-M enzyme detected in bacteria from companion animals from Europe (Coque, Baquero & Canton, 2008;

[4] Metallo-β-lactamases are those containing a zinc active site(s) (Bush, 2013).
[5] Serine-β-lactamases are those containing a serine active site(s) (Bush, 2013).

Table 5 – 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-β-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-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime and 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-β-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-β-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-β-lactams and 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.

Ewers et al., 2012). CTX-M enzymes can be found in Enterobacteriaceae, especially *E. coli* and *Klebsiella* spp. (Doi et al., 2017). It is noteworthy that there is a strong association between *E. coli* sequence type (ST) 131[6] clonal lineage, especially the O25b:H4-B2-ST131 (Nicolas-Chanoine et al., 2008), and the dissemination of CTX-M-15 (Coque et al., 2008; Doi et al., 2017). This clone is also relevant because it exhibits a wide virulence gene profile (Nicolas-Chanoine et al., 2008) and it is an important uropathogen (Vimont et al., 2012). Furthermore, O25b:H4-B2-ST131 CTX-M-15-producing *E. coli* has been reported in humans and more rarely in companion animals (Nicolas-Chanoine et al., 2008; Pomba, da Fonseca, Baptista & Correia, 2009; Ewers et al., 2010).

Although still less common than ESBLs (Jacoby, 2012; Smet et al., 2010; Ewers et al., 2012), AmpC cephalosporinases (class C, groups 1 and 1e), are also clinically relevant and may be chromosomal or plasmid encoded (Bush et al., 1995; Jacoby, 2012). These also include several enzymes families (e.g. MOX, FOC, DHA, ACC, MIR, CMY) that are more hydrolytic than ESBL, are usually resistant to clavulanic acid and active on cephamicyns (Jacoby, 2012; Bush & Jacoby, 2010). The most common AmpC cephalosporinases in humans and companion animals is CMY-2 (Smet et al., 2010; Ewers et al., 2012).

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; Quale, Bratu, Gupta & Landman, 2006; Wozniak et al., 2012).

Carbapenemases belong to β-lactamase class B (group 3a and 3b), class A and class D (group 2f and 2df, respectively), are able to hydrolyse almost all β-lactams including carbapenems and may not be inhibited by β-lactamase inhibitors (Queenan & Bush, 2007). These β-lactamases can be chromosomal or plasmid-mediated and belong to several clinically relevant enzyme families (Bush & Jacoby, 2010). Among carbapenemases, KPC, IMP, VIM, NDM and OXA are enzyme families of particular interest because of their dissemination potential since they are plasmid-mediated and can be found in several Gram-negative bacteria (Queenan & Bush, 2007). The detection of carbapenemases in companion animals is still rare, however, the number of reports of companion animals infection (including UTI) and colonisation by carbapenemase-producing Gram-negative bacteria is increasing (Shaheen, Nayak & Bootheb, 2013; Stolle et al., 2013; Pomba et al., 2014a; Schmiedel et al., 2014; Abraham et al., 2016; Ewers et al., 2016; González-Torralba et al., 2016; Hérivaux et al., 2016; Liu et al., 2016; Yousfi et al., 2016; Ewers et al., 2017; Melo et al., 2017; Yousfi et al., 2017; Chanchaithong et al., 2018; Cui et al., 2018; Daniels et al., 2018; Fernandes et al., 2018a; Grönthal et al., 2018; Hong et al., 2018; Köck et al., 2018; Pulss et al., 2018).

[6] As defined by multilocus sequence typing.

1.2.2 Thrimethoprim/sulfamethoxazole

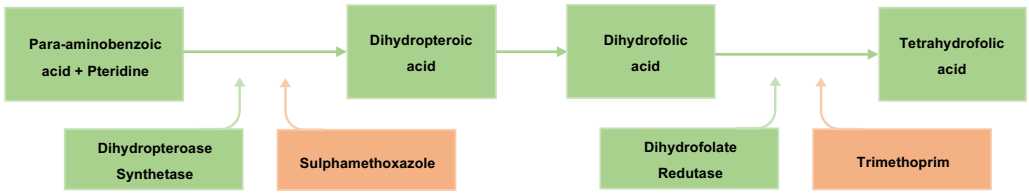
Trimethoprim and sulfonamides are synthetic antimicrobials that when in combination have a synergistic effect with broader spectrum of activity and bactericidal action (Bushby & Hitchings, 1968). These antimicrobials act at different steps of the bacteria tetrahydrofolic acid biosynthesis, which is essential for bacteria amino acid and nucleotide synthesis (Eliopoulos & Huovinen, 2001).

Discovered in the 1930s, sulfonamides competitively inhibit the enzyme dihydropteroate synthase (DHPS) at an early step of the folate synthesis pathway (Figure 3) (Sköld, 2000).

Trimethoprim competitively inhibits the enzyme dihydrofolate reductase (DHFR) at the final step of tetrahydrofolic acid synthesis and was first used in the 1960s (Huovinen, 1987).

Although trimethoprim/sulfamethoxazole is considered useful for UTI treatment in humans since 1968 (Eliopoulos & Huovinen, 2001), its use has become limited in some countries due to current high trimethoprim/sulfamethoxazole-resistance rates (EAU, 2017). The trimethoprim/sulfamethoxazole combination is considered a good first choice for UTI treatment in companion animals (Table 3) (Weese et al., 2011).

Figure 3 – Trimethoprim and sulfamethoxazole inhibition of tetrahydrofolic acid biosynthesis



Acquired trimethoprim-resistance may be related to chromosomal mutations (e.g. promoter mutations or mutations in the DHFR gene) or by the acquisition of exogenous genes encoding a DHFR with an altered active site (Eliopoulos & Huovinen, 2001). A high number of transferable trimethoprim-resistant DHFRs genes located in gene cassettes have been described and are grouped in two families (*dfrA* and *dfrB*) (White & Rawlinson, 2001).

Resistance to sulfonamides may also be caused by chromosomal mutations or plasmid borne mechanisms (Sköld, 2000). The main clinical acquired sulfonamide-resistance mechanism in Enterobacteriaceae, is the presence of DHPS with low sulfonamide affinity encoded in mobile elements (Wise & Abou-Donia, 1975; Sköld, 1976; Sköld, 2000). The first transferable sulfonamide-resistance genes, *sul1* and *sul2*, were described in the 1980s while only in 2003 *sul3* was dicovered (Rådström & Swedberg, 1988; Sundström, Rådström, Swedberg & Sköld, 1988; Perreten & Boerlin, 2003). More recently, *sul4* was noticed by next generation sequencing (Razavi et al., 2017). The *sul1* and *sul2* genes are very common among Enterobacteriaceae resistant clinical isolates (Rådström, Swedberg & Sköld, 1991; Grape, Sundström & Kronvall, 2003). In Europe, uropathogenic *E. coli* from humans with UTI have

higher frequency of *sul2* and *dfrA1* (Kerrn, Klemmensen, Frimodt-Møller & Espersen, 2002; Grape et al., 2003; Blahna et al., 2006). The *sul3* is less common (Grape et al., 2003; Blahna et al., 2006) and, due to its novelty, little is known about *sul4*.

1.2.3 Fluoroquinolones

Quinolone are synthetic antimicrobials with good oral bioavailability, a good safety profile and achieve good urinary concentrations (Andriole, 2005; Weese et al., 2011; Hooper & Jacoby, 2015). Quinolones act by interacting with DNA gyrase and topoisomerase IV bacterial enzymes that are essential to regulate the topology of the bacterial chromosome and adequate DNA replication (Andriole, 2005; Hooper & Jacoby, 2015). Both enzymes are heterotetrameric proteins composed of two subunits, *gyr*A and *gyr*B in DNA gyrase and *par*C and *par*E in topoisomerase IV (Andriole, 2005; Hooper & Jacoby, 2015). By interacting with DNA gyrase and topoisomerase IV, quinolones inhibit DNA synthesis leading to bacterial death (Andriole, 2005; Hooper & Jacoby, 2015). Quinolone affinity to both enzymes may vary depending on the quinolone and bacterial species (Hoshino et al., 1994; Blanche et al., 1996). In Gram-negative bacteria, DNA gyrase is usually the main target (Hoshino et al., 1994).

After β-lactams, quinolones are among the most sold antimicrobial in humans and companion animals (ECDC, 2016a; EMA, 2017b). Nalidixic acid was the first quinolone described in the 1960s for UTI treatment (Lesher et al., 1962). Since then, several other quinolones were discovered including for veterinary use. Quinolones can be classified into first to forth-generation based on their spectrum of activity. Nevertheless, there are slight variations in its classification system according to the author (King, Malone & Lilley, 2000; Andriole, 2005). In veterinary medicine, there are quinolones available from the first, second (e.g. marbofloxacin, enrofloxacin) and third-generation (e.g. pradofloxacin), some of which are recommended for UTI treatment (Cotard et al., 1995; Weese et al., 2011). Although not referred in Weese et al. (2011) UTI guidelines, pradofloxacin has been shown to be useful for the treatment of UTIs in cats (Litster et., 2007a). Fluoroquinolones are CIAs of highest importance for humans (WHO, 2017a) and have been shown to increase the risk for selection of resistant bacteria such as MRSA (Venezia, Domaracki, Evans, Preston & Graffunder, 2001; Weber, Gold, Hooper, Karchmer & Carmeli, 2003). Although fluoroquinolones should be regarded as second line treatment option in companion animals with UTI (Weese et al., 2011), a short duration protocol (3 days) using high dose of enrofloxacin in dogs with uncomplicated UTI has been shown to be successful (Westropp et al., 2012). Ciprofloxacin concentrations atchieved in urine with such high dose enrofloxacin protocol were considered protective against *E. coli* mutant selection in urine (Daniels, Tracy, Irom & Lakritz, 2014). Nevertheless, the impact on the commensal bacteria resistome in comparison with current first line treatment options still needs to be studied (Daniels et al., 2014).

In Gram-negative bacteria, resistance to quinolones may result from different resistance mechanisms such as topoisomerase target mutations, cell wall permeability changes, topoisomerase target protection by specific proteins and drug inactivation (Hooper & Jacoby, 2015).

Low quinolone affinity due to topoisomerase target mutations in the quinolone resistance-determining regions (QRDRs) is the main quinolone acquired resistance mechanism (Willmott & Maxwell, 1993; Barnard & Maxwell, 2001; Rodríguez-Martínez, Cano, Velasco, Martínez-Martínez & Pascual, 2011). Single topoisomerase mutations may not confer clinically significant resistance in Enterobacteriaceae. The accumulation of multiple mutations is involved in quinolone-resistance evolution and increases the resistance level (Deguchi et al., 1997; Komp Lindgren, Karlsson & Hughes, 2003). In fact, DNA gyrase and topoisomerase IV are prone to acquire an increasing number of mutations when exposed to quinolones (Jacoby, 2005). In Gram-negative bacteria, the first step mutation usually occurs in the *gyrA* and less frequently in *gyrB* gene (Nakamura, Nakamura, Kojima & Yoshida, 1989; Khodursky, Zechiedrich & Cozzarelli, 1995; Deguchi et al., 1997; Hooper & Jacoby, 2015). In these bacteria, ParC and ParE are usually the target of second-step mutations (Khodursky, Zechiedrich & Cozzarelli, 1995; Breines et al., 1997; Deguchi et al., 1997). QRDR mutations can lead to 10- to 100-fold increases in resistance (Yoshida, Kojima, Yamagishi & Nakamura, 1988; Khodursky et al., 1995).

Since the DNA gyrase and topoisomerase IV are located in the bacteria cytoplasm, changes in the cell permeability may lead to quinolone-resistance (Hirai, Aoyama, Irikura, Iyobe & Mitsuhashi, 1986; Hooper & Jacoby, 2015). Plasmid encoded active efflux pumps responsible for quinolone-resistance in Gram-negative bacteria have been reported, such as QepA and OqxAB (Hansen, Johannesen, Burmølle, Sørensen & Sørensen, 2004; Yamane et al., 2007). These increase the resistance to hydrophilic fluoroquinolones like norfloxacin, ciprofloxacin, and enrofloxacin, and to other antimicrobial classes (Hansen et al., 2004; Hansen, Jensen, Sørensen & Sørensen, 2007; Yamane et al., 2007). In *K. pneumonia*, OqxAB efflux pump may be plasmid or chromosome encoded (Kim et al., 2009c; Rodríguez-Martínez et al., 2013).

Plasmid-encoded Qnr proteins (small pentapeptide-repeat proteins) bind to the topoisomerase targets, protecting them from quinolones, therefore leading to increased resistance (Tran & Jacoby, 2002). The first plasmid-mediated Qnr determinant, QnrA, was described in 1998 in a *K. pneumoniae* (Martínez-Martínez, Pascual & Jacoby, 1998). Several additional plasmid-encoded *qnr* alleles (*qnrB*, *qnrC*, *qnrD*, *qnrS* and *qnrVC*) and multiple variants have been described since then in Gram-negative bacteria (Jacoby et al., 2008; Rodríguez-Martínez et al., 2011; Fonseca & Vicente, 2013). These proteins are disseminated worldwide in Gram-negative bacteria, however, Qnr determinants seem to still be rare except in some groups such as ESBL-producers (Hooper & Jacoby, 2015). QnrB seems to the most common (Kim et al., 2009a; Jacoby, Strahilevitz & Hooper, 2014).

Quinolone-resistance may also be caused by quinolone modification by a plasmid-encoded mutated aminoglycoside acetyltransferase (AAC(6')-Ib-cr) that evolved to acetylate quinolones such as ciprofloxacin (Robicsek et al., 2006a). This enzyme is generally plasmid encoded but has already been found encoded in the chromosome of Enterobacteriaceae (Ruiz et al., 2012). This resistance mechanism is worldwide disseminated in Enterobacteriaceae such as *E. coli* and *K. pneumoniae* and is likely the most common plasmid-mediated quinolone resistance (PMQR) determinant (Park, Robicsek, Jacoby, Sahm & Hooper, 2006; Robicsek et al., 2006a; Pitout, Wei, Church & Gregson, 2008; Kim et al., 2009b; Musumeci et al., 2012; Ruiz et al., 2012).

PMQRs are responsible for low-level quinolone resistance (Robicsek et al., 2006a; Rodríguez-Martínez et al., 2011). However, by reducing quinolone-susceptibility even at low level, PMQR determinants favour the selection of QRDR mutants evolving towards higher and clinically relevant resistance levels (Rodríguez-Martínez et al., 2011; Jacoby, 2005; Hooper & Jacoby, 2015). These resistance determinants may be encoded in plasmids harbouring ESBL and other resistance genes, thus, the use of quinolones may contribute to the co-selection of ESBLs and other resistance determinants and vice versa (Rodríguez-Martínez et al., 2011; Ruiz et al., 2011; Liu et al., 2013; Jacoby, Strahilevitz & Hooper, 2014). PMQRs are detected worldwide and have been found in Enterobacteriaceae isolated from humans and companion animals (Jacoby, Strahilevitz & Hooper, 2014).

1.2.4 Aminoglycosides

Aminoglycosides are broad spectrum bactericidal antimicrobials (except spectinomycin and kasugamycin) that interfere with protein synthesis by binding to the bacterial ribosome (16S rRNA of the 30S ribosomals subunit) (Ramirez & Tolmasky, 2010). Since the first clinical use of aminoglycosides (streptomycin) in the 1940s, several synthetic derivates have been developed with different spectrums of activity (Ramirez & Tolmasky, 2010; European Medicines Agency [EMA], 2017a). Aminoglycosides can be divided in 4 groups based on their structure (EMA, 2017a). Aminoglycosides, especially gentamicin, tobramycin and amikacin, are clinically relevant for the treatment of multidrug-resistant (MDR) Gram-negative bacteria infection and in critical care of humans (EMA, 2017a). Furthermore, aminoglycosides are CIAs of high priority to humans (WHO, 2017a). Aminoglycosides are mainly excreted by glomerular filtration in the active form (Ramirez & Tolmasky, 2010) but are not usually considered as a first line treatment option for UTI due to its nephrotoxicity side effects (Weese et al., 2011). In Weese et al. (2011) companion animal antimicrobial guideline, amikacin is recommended for the treatment of UTI by MDR bacteria if renal insufficiency is absent. It is noteworthy that gentamicin has also been shown to be useful in the treatment of UTI in dogs (Ling & Ruby, 1979).

The most common aminoglycoside resistance mechanism is the production of aminoglycoside-modifying enzymes (AMEs). Nevertheless, ribosomal target modification and changes in cell wall aminoglycoside uptake can also be involved (Garneau-Tsodikova & Labby, 2016; EMA, 2017a).

AMEs can be grouped into 3 major sub-classes according to the type of chemical modification they produce in the target aminoglycoside, namely *O*-phosphotransferases (APH), *N*-acetyltransferases (AAC) and *O*-nucleotidyltransferases (ANT) (Ramirez & Tolmasky, 2010). Each AME sub-class includes several enzymes with different spectrums of activity. Among the high number of AMEs described so far, the AAC(6')-Ib, is the most prevalent and clinically relevant AME in Gram-negative bacteria (Ramirez & Tolmasky, 2010; Ramirez, Nikolaidis & Tolmasky, 2013; Garneau-Tsodikova & Labby, 2016). Several AAC(6')-Ib enzyme variations have been described including the widely disseminated ciprofloxacin modifying variant AAC(6')-Ib-cr (Ramirez et al., 2013). Some AMEs may act on aminoglycosides by two different reactions (Ramirez & Tolmasky, 2010). The bifunctional enzyme (AAC(6′)-Ie-APH(2″)-Ia), is of clinical importance in *Enterococcus* spp. because it confers high-level resistance to most aminoglycosides precluding the synergistic action with β-lactams (Chow, 2000). AMEs are often encoded in mobile elements such as plasmids and may be associated with additional resistance genes towards other antimicrobials (Garneau-Tsodikova & Labby, 2016).

Aminoglycosides target the A-site of bacterial ribosome, so the modification of the aminoglycoside ribosomal binding site may lead to aminoglycoside-resistance (Garneau-Tsodikova & Labby, 2016). Modification of the ribosomal binding site can be caused by ribosomal protein mutation; however, these mutations are rare (Garneau-Tsodikova & Labby, 2016). The ribosomal binding site may also be modified enzymatically by 16S ribosomal RNA methyltransferases (16S rRNA methylases) (Wachino & Arakawa, 2012). The 16S rRNA methylases can be classified into two families according to the nucleotide position where methylation occur (Garneau-Tsodikova & Labby, 2016). The 16S rRNA methylases may have a broader substrate than AMEs. Since these enzymes can be plasmid-mediated in Gram-negative bacteria, its dissemination is a concern (Wachino & Arakawa, 2012). These enzymes have been reported worldwide in Enterobacteriaceae, with RtmB and ArmA as the most common. Although 16S rRNA methylases frequency is still low, reports are becoming more common including in companion animals (Deng et al., 2011; Wachino & Arakawa, 2012; Hidalgo et al., 2013; Xia et al., 2017).

To reach the cytoplasm of Gram-negative bacteria aminoglycosides first need to transverse the cell wall. Low aminoglycoside uptake leading to resistance may result from porin changes, LPS charge modifications and presence of efflux systems (Garneau-Tsodikova & Labby, 2016).

1.2.5 Tetracycline

Tetracyclines are bacteriostatic broad-spectrum antimicrobials that bind to the 30S ribosomal subunit (A-site) and inhibit bacterial protein synthesis (Chopra & Roberts, 2001). Among tetracyclines, doxycicline may be used for the treatment of UTIs in humans and companion animals (Weese et al., 2011; Wilso et al., 2006; EAU, 2017). However, is not considered a first line option (Weese et al., 2011).

With the widespread use of tetracyclines after their discovery in the 1940s, resistance started to increase (Chopra & Roberts, 2001). In Gram-negative bacteria, clinical tetracycline-resistance may result from active efflux systems or from ribosomal target protection.

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 & Roberts, 2001; Roberts, 2017). Most of these efflux systems are responsible for resistance to tetracycline but not to minocycline or tigecycline (Chopra & Roberts, 2001), except tet(B) that is also responsible for minocycline-resistance. These are frequently encoded in conjugative plasmids harbouring resistance genes to several other antimicrobials (Chopra & Roberts, 2001).

Tetracyclines can also be inhibited by the production of cytoplasmic proteins that protect the ribosome. Most ribosomal protection proteins are encoded by *tet* genes and are responsible for resistance to tetracyclines, doxycicline and minocycline (Chopra & Roberts, 2001).

1.2.6 Nitrofurantoin

Nitrofurantoin is a synthetic, bactericidal, broad-spectrum activity antimicrobial that it is effective against most Gram-positive and Gram-negative bacteria (Huttner et al., 2015).

Nitrofurantoin is an old antimicrobial, discovered in the 1940s, that resurged due to the increase in antimicrobial resistance to other antimicrobial classes and the emergence of important resistance mechanisms such as ESBLs (Huttner et al., 2015). Currently, nitrofurantoin is considered one of the first option antimicrobials for the treatment of uncomplicated UTIs in humans (EAU, 2017). Despite only concentrating well in the lower urinary tract, nitrofurantoin showed equivalent efficacy to newer antimicrobials when prescribed for 5-7 days (Huttner et al., 2015). Furthermore, nitrofurantoin has the advantage of not being prone to become resistant nor to select resistance to other antimicrobials such as, for example, fluoroquinolones (Gupta, Hooton & Stamm, 2005; Sandegren, Lindqvist, Kahlmeter & Andersson, 2008; Huttner et al., 2015). Resistance to nitrofurantoin seems to be associated with chromosomal mutations, nevertheless, the OqxAB plasmid-mediated efflux pump may contribute to nitrofurantoin-resistance in *E. coli* (Ho et al., 2016).

Nitrofurantoin is not approved for veterinary use; however, its off-lable use is recommended by Weese et al. (2011) guidelines as a second line option for the treatment of MDR uncomplicated UTIs in companion animals. There is a lack of studies reporting the

concentration of nitrofurantoin achieved in urine in companion animals, nevertheless the successful use of nitrofurantoin in the treatment of a cat with UTI has been reported (Pomba, Couto & Moodley, 2010).

1.2.7 Fosfomycin

Fosfomycin is a bactericidal broad-spectrum antimicrobial discovered in the 1960s that inhibits cell wall and early murein/peptidoglycan synthesis (Kahan, Kahan, Cassidy & Kropp, 1974; Pérez, Tapia & Soraci, 2014). Fosfomycin may have synergistic effect with other antimicrobials including in infections by *E. coli* and *K. pneumoniae* (Pérez el al., 2014). In humans, fosfomycin is excreted mainly in urine in the active form and is considered a first line option for the treatment of uncomplicated cystitis (Pérez el al., 2014; EAU, 2017). This antimicrobial is not included in Weese et al. (2011) guidelines for companion animals with UTI. In cats, but not in dogs, the use of fosfomycin seems to induce renal changes leading to kidney insufficiency (Fukata, Imai & Shibata, 2008). So far, only few studies have been conducted regarding the use of fosfomycin in dogs, none of which was focussed on UTI (Gutierrez, Ocampo, Aguilera, Luna & Sumano, 2008; Fukata et al., 2008).

Several fosfomycin-resistance mechanisms have been described involving decreased antimicrobial uptake, modification of the target site and antimicrobial inactivation (Karageorgopoulos, Wang, Yu & Falagas, 2012). Several of these resistance mechanisms result from chromosomal mutations, nonetheless some inactivation enzymes may be plasmid-mediated, such as *fosA* and *fosB* (Karageorgopoulos et al., 2012*). In vitro* fosfomycin mutational rate is high, however the same does not seem to be true *in vivo* (Karageorgopoulos et al., 2012). In agreement, fosfomycin-resistance rates seem to be relatively stable, even in countries in which fosfomycin is systemically prescribed (Karageorgopoulos et al., 2012). Plasmid-mediated fosfomycin-resistance mechanisms have been detected in human and companion animal Enterobacteriaceae (Mendes et al., 2016; Yao et al., 2016).

1.2.8 Choramphenicol and florfenicol

Chloramphenicol and florfenicol are bacteriostatic, broad-spectrum antimicrobials that inhibit protein synthesis by binding to the 50S ribosomal subunit (Schwarz, Kehrenberg, Doublet & Cloeckaert, 2004). Chloramphenicol is used in human and veterinary medicine while florfenicol is licensed only for veterinary use in farm animals (Schwarz et al., 2004). Due to the occurrence of side effects such as aplastic anaemia in humans, chloramphenicol is reserved for MDR life-threatening infections (Schwarz et al., 2004). Chloramphenicol is reserved in Weese et al. (2011) guidelines for the treatment of UTI caused by MDR bacteria in companion animals. However, care must be taken with adverse side effects (Weese et al., 2011).

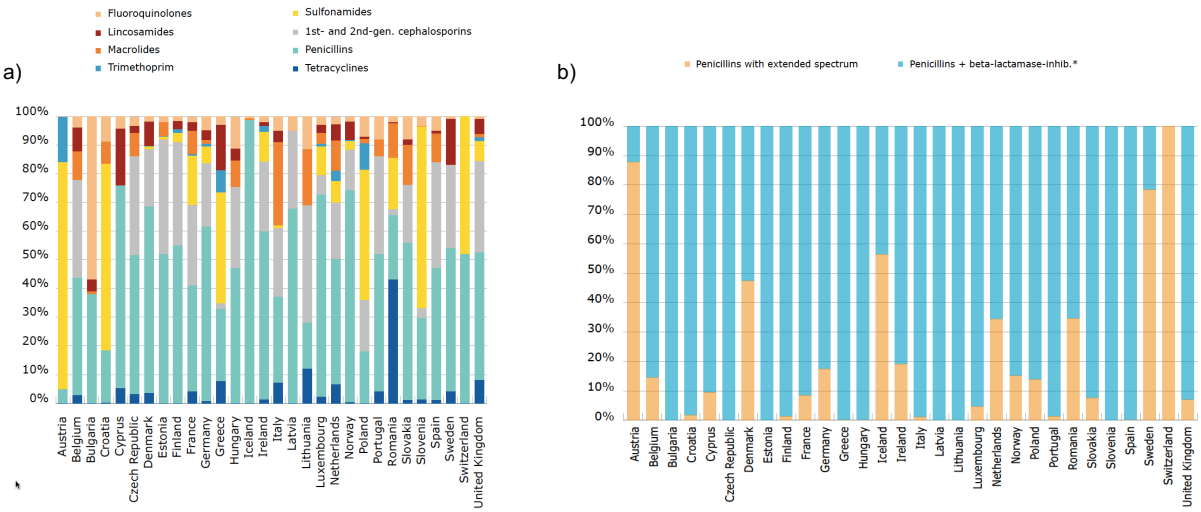
The most common resistance mechanism to chloramphenicol is caused by antimicrobial inactivation by chloramphenicol acetyltransferase (CAT) enzymes. Florfenicol is not

inactivated by CAT enzymes (Schwarz et al., 2004). Resistance may also be caused by other mechanisms such as efflux systems and target mutations. Combined resistance to chloramphenicol and florfenicol can be caused by plasmid-mediated efflux systems such as *floR*. This latter has been mostly described in Enterobacteriaceae from farm animals, but reports from companion animals and humans have also been done (Schwarz et al., 2004; Derakhshandeh et al., 2018)

1.3 Antimicrobial resistance surveillance in uropathogenic bacteria from companion animals

Updated antimicrobial resistance data is crucial to guide empirical antimicrobial treatment and to evaluate antimicrobial resistance trends. The use of antimicrobials contributes to increasing resistance trends, therefore data on antimicrobial usage is also of great importance (Guardabassi et al., 2004; Prescott, 2017). Only recently, since 2010, the European Medicine Agency started reporting data on antimicrobial sales for companion animals (EMA, 2017b). Data revealed that β-lactams, including potentiated penicillins, are the most frequently sold for companion animals in most European countries (EMA, 2017b). Nevertheless, there are profound antimicrobial sales differences between countries (Figure 4).

Figure 4 – Antimicrobial sales in companion animals (adapted from EMA, 2017b)



Legend: a) distribution of sales of antimicrobial tablets, in tonnes of active ingredient, by antimicrobial class, by country, for 2015; b) distribution of sales (by weight of active ingredient) of tablets containing penicillins by subclass, by country, in 2015.

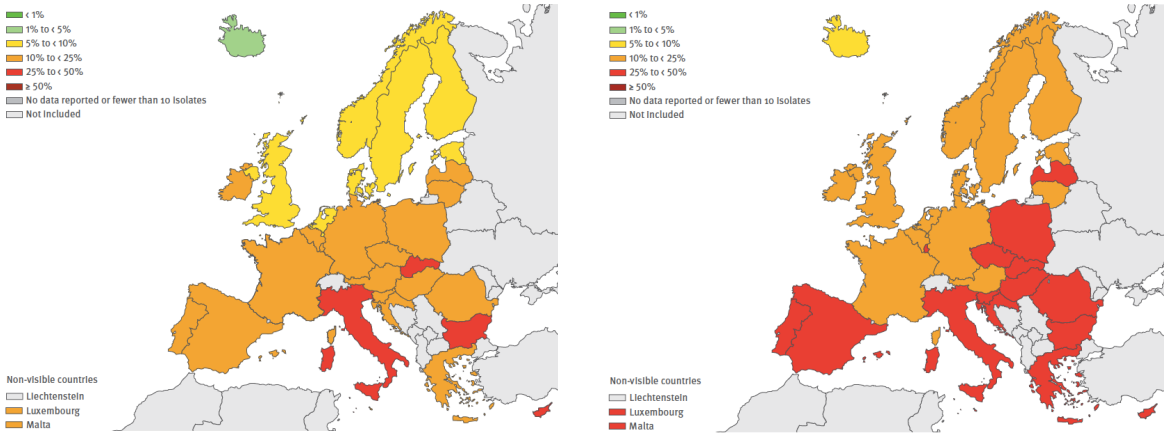
In human medicine, the European Antimicrobial Resistance Surveillance Network (EARS-Net) actively gathers and reports annual data on antimicrobial resistance of invasive bacteria from several countries in Europe (European Centre for Disease Prevention and Control [ECDC], 2017). EARS-Net reports have shown striking geographical variations in antimicrobial resistance frequencies as well as increasing resistance temporal trends to CIAs (ECDC, 2017)

(Figure 5). Studies focussed on UTI in humans also show geographical differences in antimicrobial resistance frequencies (Kahlmeter & ECO.SENS, 2003).

There have been some 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 (de Jong et al., 2013; Moyaert et al., 2017). Results from Moyaert et al. (2017) revealed overall high susceptibility to all tested antimicrobials (e.g. >90% for most antimicrobials in *E. coli*). Since results are presented for all countries as a group and no temporal trends were analysed, it is not possible to perceive any geographical or temporal antimicrobial resistance variations in the 10 included European countries.

Comparison of published data is frequently difficult because studies report antimicrobial resistance frequencies using different inclusion criteria (e.g. diabetic animals, recurrent UTI, etc.) (Bailiff et al., 2006), different time periods, grouping results of different sites of infection (Normand, Gibson, Reid, Carmichael & Taylor, 2000a; Normand, Gibson, Taylor, Carmichael & Reid, 2000b; Authier, Paquette, Labrecque & Messier, 2006; Pedersen et al., 2007; Harada, Niina, Nakai, Kataoka & Takahashi, 2012a; Beever et al., 2015), combining different bacteria genera (Ball, Rubin, Chirino-Trejo & Dowling, 2008; Hall, Holmes & Baines, 2013; Dorsch et al., 2015; Wong, Epstein & Westropp, 2015; Rampacci et al., 2018; Teichmann-Knorrn et al., 2018) and data from several countries (Meunier, Acar, Martel, Kroemer & Valle, 2004; Kroemer et al., 2014; Moyaert et al., 2017).

Figure 5 – *E. coli* antimicrobial resistance of invasive isolates from humans, by country (adapted from ECDC, 2017)



Legend: Left image, third-generation cephalosporins; Right image, fluoroquinolones.

Therefore, the analysis of the geographical and temporal distribution of antimicrobial resistance in companion animals with UTI is limited. Furthermore, little is known about Portugal since only early studies report data from companion animals with UTI (Féria, Correia, Machado, Vidal & Gonçalves, 2000; Féria, Ferreira, Correia, Gonçalves & Caniça, 2002).

Kroemer et al. (2014) European study has found lower antimicrobial susceptibility rates among *E. coli* and *P. mirabilis* from companion animals with UTI isolated in 2002-2009 than Moyaert et al. (2017). Notably, *P. mirabilis* showed high trimethoprim/sulfamethoxazol-resistance (~53%) (Kroemer et al., 2014). Overall high antimicrobial susceptibility frequencies were also detected in Sweden (2009, 2014) (Swedres-Svarm, 2016; Windahl, Holst, Nyman, Grönlund & Bengtsson, 2014), Norway (2003-2009) (Lund, Skogtun, Sørum & Eggertsdóttir, 2014) and Switzerland (*E. coli*, 1999-2001) (Lanz, Kuhnert & Boerlin, 2003). Nevertheless, most studies show varying antimicrobial resistance frequencies. Higher levels of resistance to some antimicrobials in bacteria from companion animals with UTI have 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, de Castro, Iovine, Irino & Carvalho, 2014), Cornell USA (e.g. *E. coli* ~35% to ampicillin, ~70% to cephalotin, ~20% to enrofloxacin, ~40% to gentamicin) (Cummings, Aprea & Altier, 2015); Taiwan (*E. coli* 50% amoxicillin, 39% trimethoprim/sulfamethoxazole, 2010-2011) (Chang, Lo, Wei & Kuo, 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, Zurfluh, Schmitt, Nüesch-Inderbinen & Stephan, 2018b), Belgium (e.g. *E. coli* 12% to amoxicillin/clavulanate, 17% to enrofloxacin, 2010-2012) (Criel, Steenbergen & Stalpaert, 2015), Virginia USA (*E. coli*, 18% to amoxicillin/clavulanate, 15% to trimethoprim/sulfamethoxazole,1986-1996) (Forrester et al., 1999) and Italy (2013-3015) (Rampacci et al., 2018). Furthermore, changing antimicrobial resistance trends in bacteria isolated from companion animals have been reported (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, Singer, Jang & Hirsh, 2002), Canada (fluoroquinolones, 1984-1998; several antimicrobials, 2002-2007) (Prescott et al., 2002; Ball et al., 2008), United Kingdom (enrofloxacin, cephalexin and oxytetracicline, 1999-2009) (Hall et al., 2013) and New Zealand (amoxicillin/clavulanate, cephalotin, enrofloxacin, 2005-2012) (McMeekin, Hill, Gibson, Bridges & Benschop, 2016).

Care must be taken when comparing different studies since differences in the inclusion criteria may affect antimicrobial resistance frequencies. For instance, studies from referral hospitals or recurrent UTIs (patients that have likely undergone prior antimicrobial treatments) may have a bias towards resistance when compared to studies focussed on first opinion patients (Wong et al., 2015). Another important limiting factor in comparing studies is the use of different susceptibility testing interpretation criteria over time, since clinical breakpoints are regularly updated (Hombach, Bloemberg & Böttger, 2012). Currently, the most frequently used clinical breakpoints are the ones proposed by the Clinical and Laboratory Standards Institute (CLSI)

and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Only CLSI has published a limited number of specific breakpoints for animals (Toutain et al., 2017). Nevertheless, since 2015 the EUCAST created a subcommittee, VetCAST, that will also propose specific clinical breakpoints for animals in the future (Toutain et al., 2017).

The detection of MDR bacteria in companion animals creates important therapeutic limitations and raises public health concerns. MDR bacteria have been widely detected in companion animals with UTI and may be associated with clinically relevant and mobile resistance mechanisms such as ESBLs and carbapenemases in Enterobacteriaceae and the *mecA* gene in staphylococci (Prescott et al., 2002; Harada et al., 2012a; Pomba et al., 2014a; Osugui et al., 2014; Wagner, Gally & Argyle, 2014; Windahl et al., 2014; Chang et al., 2015; Thungrat, Price, Carpenter & Boothe, 2015; Wong et al., 2015; Couto et al., 2016; Zogg et al., 2018b).

The definition of MDR bacteria varies significantly among studies, which impares straightforward comparisons. This issue also occurs in studies of bacteria from humans and prompted Magiorakos et al. (2012) through a joint initiative by the European Centre for Disease Prevention and Control and the Centres for Disease Control and Prevention to propose a standartised definition of MDR, extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria. These concepts were recently adapted for companion animal isolates aiming to also harmonise future antimicrobial resistance surveillance reports in veterinary medicine (Sweeney, Lubbers, Schwarz & Watts, 2018).

1.4 Klebsiella pneumoniae

Klebsiella species are ubiquitous, non-motile, encapsulated Gram-negative Enterobacteriaceae that can be found in nature (e.g. plants and soil) and colonising healthy animals and humans (Podschun & Ullmann, 1998; Brisse et al., 2006). *Klebsiella* species can cause several types of infections in humans and companion animals and *K. pneumoniae* is the most frequently identified species (Podschun & Ullmann, 1998; Brisse et al., 2006; Ewers et al., 2014b, Paczosa & Mecsas, 2016).

K. pneumoniae was first described in 1882 by Carl Friedlander from a patient with pneumonia (Friedlaender, 1882). *K. pneumoniae* is an important worldwide ESKAPE[7] nosocomial pathogen, which is responsible for ~8-9% of nosocomial infections in the United States of America and Europe (Rice, 2008; ECDC, 2013; Magill et al., 2014). The frequent *K. pneumoniae* MDR profile and association with ESBLs and carbapenemase β-lactamases (Brisse et al., 2006; ECDC, 2017; Navon-Venezia, Kondratyeva & Carattoli, 2017), leads to big therapeutic limitations. The urinary tract is among the most common sites of nosocomial infection by *K. pneumoniae* in humans (Podschun & Ullmann, 1998). Also, *K. pneumoniae* is the second most common pathogen in women with uncomplicated UTI

[7] ESKAPE is an acronym encompassing the names of six bacterial pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp..

(Gupta et al., 1999). Importatly, *K. pneumoniae* are prone to nosocomial dissemination which increases their public health relevance (Brisse et al., 2006).

K. pneumoniae is frequently referred to as an opportunistic agent since usually affects hospitalised or immunocompromised patients (Podschun & Ullmann, 1998). More recently, new hypervirulent *K. pneumoniae* strains able to cause severe infections in otherwise healthy patients are being increasingly reported (Shon, Bajwa & Russo, 2013; Paczosa & Mecsas, 2016).

1.4.1 Population structure

Since its discovery, several *Klebsiella* species have been described and its taxonomy has been continuously revised due to novel scientific findings (Podschun & Ullmann, 1998; Brisse, Grimont & Grimont, 2006; Brisse, Passet & Grimont, 2014). According to the Ørskov classification, adopted in America and most European countries, *K. pneumoniae* includes three subspecies, namely *Klebsiella pneumoniae* subspecies *pneumoniae*, *Klebsiella pneumoniae* subspecies *ozaenae* and *Klebsiella pneumoniae* subspecies *rhinoscleromatis* (Ørskov 1984[8] cited by Podschun & Ullmann, 1998).

Based on several phylogenetic molecular markers, *K. pneumoniae* subspecies were shown to be closely related in agreement with Ørskov classification (Brisse & Verhoef, 2001; Drancourt, Bollet, Carta & Rousselier, 2001; Brisse et al., 2014). Although it is still unknown, sequencing data suggests that *Klebsiella granulomatis* is also a *K. pneumoniae* subspecies (Drancourt et al., 2001; Brisse et al., 2006).

In Brisse and Verhoef (2001) study, strains classically identified as *K. pneumoniae* appeared to be composed of three major clusters (KpI, KpII and KpIII). These clusters did not correspond to the known taxonomic *K. pneumoniae* subspecies since these latter all belonged to KpI (Brisse & Verhoef, 2001). Latter studies have clarified that KpI indeed includes the three *K. pneumoniae* subspecies while KpII and KpIII are distinct *Klebsiella* species (Brisse et al., 2014). Cluster KpII was shown to contain a novel *Klebsiella* species, *Klebsiella quasipneumoniae*, that includes clusters KpII-A and KpII-B, corresponding to two subspecies (*K. quasipneumoniae subspecies quasipneumoniae and K. quasipneumoniae* subspecies *similipneumoniae*, respectively) (Brisse et al., 2014). KpIII corresponded to *Klebsiella variicola* (syn. *Klebsiella singaporensis*) first described in 2004 (Rosenblueth, Martínez, Silva & Martínez-Romero, 2004; Brisse et al., 2014). Due to its recent discovery, not much is yet known about KpII or KpIII epidemiology and previous studies may have misidentified them with *K. pneumoniae* (KpI). Using strains from several European hospitals, Brisse, van Himbergen, Kusters and Verhoef (2004b) showed that cluster KpI (corresponding to *K. pneumoniae*) is responsible for 80% of hospital infections, followed by cluster KpIII and KpII. Nevertheless,

[8] Ørskov, I. (1984). Genus *Klebsiella*. In: Krieg N.R. & Holt J.G. (ed.) Bergey's manual of systematic bacteriology. Baltimore: The Williams & Wilkins Co..

KpI, KpII and KpIII may all be associated with UTIs in humans (Long et al., 2017). In animal infections, KpI is also the most common cluster, followed by KpIII and KpII (Brisse & Duijkeren, 2005). KpI, KpII and KpIII have been detected in infected dogs and only KpI has been reported in infected cats (Brisse & Duijkeren, 2005; Brisse et al., 2014; Harada et al., 2016). Furthermore, KpI strains have been isolated from companion animals with UTI (Brisse & Duijkeren, 2005; Harada et al., 2016).

The analysis of the *K. pneumoniae* population structure is essential to assist in epidemiological investigations and to recognise clonal groups and their links to pathophysiological specificities (Brisse et al., 2013). Early *K. pneumoniae* typing was conducted using phenotypic features such as biotyping, serotyping, phage typing and bacteriocin typing (Podschun & Ullmann, 1998). However, most of these techniques did not allow true evaluation of epidemiological relatedness among *K. pneumoniae* isolates (Podschun & Ullmann, 1998; Brisse et al., 2006). With the arising and evolution of molecular typing methods, higher discriminatory power was achieved (Brisse et al., 2006). Several molecular techniques are currently used, and the most relevant will be given in more detail.

1.4.1.1 K-typing

Typing of *Klebsiella* spp. can be based on O and K-antigen characterisation, that was originally conducted by serotyping (Podschun & Ullmann, 1998; Brisse et al., 2006). Due to the low number of *Klebsiella* spp. O-types described, K-typing (syn. capsular typing) is considered more useful for epidemiology studies (Brisse et al., 2006). Typically, *Klebsiella* spp. have well developed polysaccharide capsules that are responsible for their mucoid appearance (Podschun & Ullmann, 1998). To date, a total of 78 K-antigens are recognised internationally (Ørskov & Fife-Asbury, 1977; Pan et al., 2008). K-typing is highly discriminatory (Hansen, Skov, Benedí, Sperling & Kolmos, 2002), however, since for a long period it required the use of non-commercially available specific anti-sera, the technique was only available in reference centres (Podschun & Ullmann, 1998; Brisse et al., 2006). To overcome this limitation, Brisse, Issenhuth-Jeanjean & Grimont (2004a) devised a K-typing method using restriction fragment length polymorphism (RFLP) after amplification of the *cps* gene locus which is responsible for the synthesis of the K-antigens. Furthermore, allelic-specific PCRs (polymerase chain reaction) were also developed to detect clinically relevant K-antigens (Brisse et al., 2013; Pan et al., 2013). Although more practical than classical serotyping, Brisse et al. (2004a) method is still complex and the allelic-specific PCRs only cover a limited number of K-antigens (Brisse et al., 2013). More recently, Brisse et al. (2013) developed a sequenced-based K-typing method supported by the analysis of polymorphisms of one *cps* locus conserved gene, *wz*i. This new method allowed good predictability of K-antigens and higher discriminatory power (Brisse et al., 2013). The *wzi* alleles are available and curated as part of a free on-line database allowing international comparability of results (Institut Pasteur Bigsdb, 2018). Similarly, Pan et

al. (2013) developed a *K. pneumoniae* capsular genotyping method based on sequencing of the *wzc* gene of the *cps* locus. In 2016, a new molecular typing method relying on whole-genome sequencing was proposed in which the entire *cps* locus (K-locus) is included (Wyres et al., 2016). In this study, the K-locus typing nomenclature was defined and an on-line database (*Kaptive*) was launched, assuring that the new K-locus typing system is comparable worldwide (Wyres et al., 2016).

1.4.1.2 Pulse field gel electrophoresis

Pulse field gel electrophoreses (PFGE) relies on the cleavage of highly purified genomic bacterial DNA with a restriction endonuclease, followed by separation of the resulting large DNA fragments in an agarose gel electrophoresis in which the electric field changes periodically (Sabat et al., 2013). Comparison of the resulting restriction pattern is used to infer the relatedness between isolates (Tenover et al., 1995). For many years, PFGE has been one of the most used DNA fingerprint methods for bacterial typing, including for *Klebsiella* spp. (Sabat et al., 2013**;** Brisse et al., 2006).

Among the advantages of this technique is that it uses a large portion of the genome (>90%), it has high concordance with epidemiological relatedness, it has excellent discriminatory power, good typeability and intra-laboratory reproducibility (Sabat et al., 2013; Centres for Disease Control and Prevention [CDC], 2016). However, this technique suffers from some pitfalls, such as being technically demanding, labour-intensive and time-consuming (Sabat et al., 2013). Also, it is important to notice that PFGE may not distinguish bands of nearly identical size and that similar sized bands between strains are not necessarily equal in genomic content. Moreover, PFGE restriction patterns may slightly vary between laboratories and the analysis of PFGE patterns is somewhat subjective (Tenover et al., 1995; Sabat et al., 2013; CDC, 2016).

To improve inter-laboratory comparisons, PFGE protocols for several bacteria were standardised through initiatives, such as PulseNet (CDC, 2016). However, there is no standardised PFGE protocol for *K. pneumoniae* (CDC, 2016) leading to difficult inter-laboratory/study comparability.

In *Klebsiella* species, the restriction enzymes include *Hae*III, *Xba*I or *Spe*I, which have been mainly used to characterise *K. pneumoniae* outbreaks (Brisse et al., 2006). When compared with K-typing, ribotyping or multilocus sequence typing (MLST), PFGE has higher discriminatory power (Hansen et al., 2002; Vimont, Mnif, Fevre & Brisse, 2008). However, PFGE has lower standardisation and inter-laboratory reproducibility then K-typing and MLST (Hansen et al., 2002; Vilmont et al., 2008).

In companion animals, PFGE has been used in studies with selected *K. pneumoniae* samples such as ESBL-, carbapenemase- and 16S rRNA methylase-producers (Haenni, Ponsin, Métayer, Médaille & Madec, 2012; Hidalgo et al., 2013; Stolle et al., 2013; Donati et al., 2014;

Ewers et al., 2014b; Wohlwend, Endimiani, Francey & Perreten, 2015; Harada et al., 2016; Xia et al., 2017). Like in human hospitals, some studies in companion animals highlighted the *K. pneumoniae* predisposition for nosocomial dissemination (Haenni et al., 2012; Ewers et al., 2014b; Wohlwend et al., 2015; Harada et al., 2016; Xia et al., 2017). Only few of these studies, using a limited number of isolates, have also included strains from human infection (Stolle et al., 2013; Ewers et al., 2014b; Wohlwend et al., 2015). Ewers et al. (2014b) detected high similarity between some human and animal strains. However, the same was not true in Wohlwend et al. (2015) study. Most studies included at least one *K. pneumoniae* from UTI (Haenni et al., 2012; Hidalgo et al., 2013; Stolle et al., 2013; Donati et al., 2014; Ewers et al., 2014b; Wohlwend et al., 2015; Xia et al., 2017), however none of them was focussed on UTI epidemiology in companion animals.

1.4.1.3 Multilocus sequence typing

MLST is based on the sequencing and comparison of several housekeeping genes (Maiden et al., 2013; Sabat et al., 2013). All unique allele sequences of each locus are assigned arbitrary numbers and the combination of alleles in an isolate determines its sequence type (ST) (Maiden et al., 2013; Sabat et al., 2013). The housekeeping genes considered vary according to the bacteria species (Sabat et al., 2013). Diancourt, Passet, Verhoef, Grimont and Brisse (2005) developed a *K. pneumoniae* MLST scheme based on *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB* housekeeping genes. All allele and ST numbering is standardised and available in an international database (Institut Pasteur Bigsdb, 2018). Therefore, MLST has the advantage of being comparable worldwide, unambiguous and highly reproducible (Sabat et al., 2013). Although the number of allelic sequence differences is not considered, the comparison of the allelic profiles among known STs allows to determine their genetic relatedness (Sabat et al., 2013). Of note, MLST is less discriminatory than PFGE (Vilmont et al., 2008). In 2005, Diancourt et al. (2005) described 40 different STs, hence, pointing to a high diversity within *K. pneumoniae*. Currently, more than 3000 STs have been designated (Institut Pasteur Bigsdb, 2018).

In companion animals several studies have determined the *K. pneumoniae* clonal lineages through MLST (Table 6). Although most studies include a varying number of *K. pneumoniae* isolated from companion animal's urine, MLST typing is mostly conducted in selected bacteria such as ESBL-producers. Poirel et al. (2013) found that urine and faecal *K. pneumoniae* from animals in France belonged mainly to ST274, while in human faecal and urine samples a higher number of STs could be found. Higher clonal diversity among human *K. pneumoniae* was also noticed by Wohlwend et al. (2015) in Switzerland, although nosocomial spread of *K. pneumoniae* strains among the included companion animals was suspected. Overall, the *K. pneumoniae* ST15 seems to be the most frequent ST in companion animals and has been detected worldwide (Table 6).

Table 6 – Sequence types described in *K. pneumoniae* from companion animals

Study	Year	Total[a] number	Clones (number)	Country	Source (number)	Species	Choice reasoning
Haenni et al., 2012	2008-2010	*n*= 24	ST15 (24)	Paris	Urine (24)	Cat and dog	ESBL-producers
Poirel et al., 2013	2011-2012	*n*= 11	ST 15 (1)	Paris	Urine (1)	Dog	ESBL/carbapenemase-producers
			ST274 (10)	Paris	Urine (5) and faeces	Cat and dog	ESBL/carbapenemase-producers
Stolle et al., 2013	2012	*n*= 5	ST15 (5)	Germany	Varied (urine, 1)	Dog	Carbapenemase-producers
Hidalgo et al., 2013	2008-2010	*n*= 7	ST11 (7)	Spain	Abcess and urine (6)	Cat and dog	Aminoglycoside-resistant
Ewers et al., 2014b	2008-2010	*n*= 65	ST70 (2)	Germany	Swab trachea	Cat and dog	ESBL-producers
			ST15 (60)	Spain, Germany, Italy, Denmark and Netherlands	Swab wound, swab trachea, faeces and Uncertain	Cat and dog	ESBL-producers
			ST188 (1)	Luxemburg	Swab trachea	Dog	ESBL-producers
			ST989 (1)	Italy	Urine (1)	Cat	ESBL-producers
			ST991 (1)	Germany	Faeces	Dog	ESBL-producers
Donati et al., 2014	2006-2012	*n*= 15	ST11 (1)	Italy	Dermatitis	Dog	ESBL-producers
			ST15 (4)	Italy	Pneumonia, otitis and septicemia	Cat and dog	ESBL-producers
			ST101 (8)	Italy	Varied (urine, 2)	Cat and dog	ESBL-producers
			ST340 (2)	Italy	Intracardiac clot	Cat and dog	ESBL-producers
Wohlwend et al., 2015	2006-2012	*n*= 25	ST11 (21)	Switzerland	Varied (urine, 8)	Cat and dog	ESBL-producers
			ST1463 (4)	Switzerland	Urine	Dog	ESBL-producers

Table 6 (continuation) - Sequence types described in *K. pneumoniae* from companion animals

Study	Year	Total[a] number	Clones (number)	Country	Source (number)	Species	Choice reasoning
Harada et al., 2016	2003-2015	*n*= 34	ST15 (16)	Japan	Varied (urine, 11)	Cat and dog	ESBL-producers
			ST34 (1)	Japan	Urine (1)	Dog	ESBL-producers
			ST37 (1)	Japan	Oral cavity	Dog	ESBL-producers
			ST147 (1)	Japan	Urine	Dog	ESBL-producers
			ST301 (3)	Japan	Pus and urine (2)	Cat and dog	ESBL-producers
			ST337 (1)	Japan	Skin	Cat	ESBL-producers
			ST655 (5)	Japan	Varied (urine, 1)	Cat and dog	ESBL-producers
			ST709 (1)	Japan	Urine (1)	Dog	ESBL-producers
			ST753 (1)	Japan	Ascites fluid	Dog	ESBL-producers
			ST881 (1)	Japan	Urine (1)	Dog	ESBL-producers
			ST1844 (2)	Japan	Urine (2)	Dog	ESBL-producers
			ST2173 (1)	Japan	Urine (1)	Dog	ESBL-producers
González-Torralba et al., 2016	2014-2015	*n*= 1	ST2090 (1)	Spain	Rectal swab	Dog	Carbapenemase-producers
Kuan et al., 2016	2011-2013	*n*= 5	ST138 (1)	Taiwan	Urine (1)	Dog	ESBL-producers
			ST750 (1)	Taiwan	Urine (1)	Cat	ESBL-producers
			ST709 (1)	Taiwan	Urine (1)	Cat	ESBL-producers
			ST15 (1)	Taiwan	Urine (1)	Dog	ESBL-producers
			ST11 (1)	Taiwan	Urine (1)	Dog	ESBL-producers
Overejo et al., 2017	2010	*n*= 2	ST11 (1)	Spain	Uncertain	Dog	Tigecycline-resistant
			ST147 (1)	Spain	Urine (1)	Dog	Tigecycline-resistant

Table 6 (continuation) - Sequence types described in *K. pneumoniae* from companion animals

Study	Year	Total[a] number	Clones (number)	Country	Source (number)	Species	Choice reasoning
Xia et al., 2017	2010-2012	*n*= 12	ST37 (9)	China	Uncertain	Cat and dog	16S rRNA methylase
			ST147 (1)	China	Uncertain	Cat	16S rRNA methylase
			ST395 (1)	China	Uncertain	Cat	16S rRNA methylase
			ST2018 (1)	China	Uncertain	Dog	16S rRNA methylase
Taniguchi et al., 2017	2016	*n*= 1	ST37 (1)	Japan	Urine (1)	Dog	Colistin/tigecycline-resistant
Sato et al., 2018	2003-2016	*n*= 7[b]	ST11 (1)	Japan	Urine (1)	Dog	Tigecycline-non-susceptible
			ST32 (1)	Japan	Ear discharge	Dog	Tigecycline-non-susceptible
			ST15 (1)	Japan	Urine	Dog	Tigecycline-non-susceptible
			ST314 (1)	Japan	Utereus	Dog	Tigecycline-non-susceptible
			ST655 (1)	Japan	Nasal cavity	Dog	Tigecycline-non-susceptible
			ST1043 (2)	Japan	Urine (2)	Dog	Tigecycline-non-susceptible
Maeyama et al., 2018	2016	*n*= 21	ST15 (7)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST655 (3)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST11 (2)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST147 (2)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST307 (2)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST709 (2)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST4 (1)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST37 (1)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
			ST45 (1)	Japan	Uncertain	Uncertain	ESBL/AmpC-producers
Zhang et al., 2018	2015-2016	*n*= 2	ST711 (2)	Canada	Uncertain	Dog	ESBL-producers

Table 6 (continuation) - Sequence types described in *K. pneumoniae* from companion animals

Study	Year	Total[a] number	Clones (number)	Country	Source (number)	Species	Choice reasoning
Zogg et al., 2018a	2012-2016	*n*= 11	ST11(1)	Switzerland	Abscess	Dog	ESBL-producers
			ST15 (3)	Switzerland	Urine (1) and wound	Cat and dog	ESBL-producers
			ST147 (6)	Switzerland	Urine (5) and other	Cat and dog	ESBL-producers
			ST778 (1)	Switzerland	Urine (1)	Dog	ESBL-producers
Pulss et al., 2018	2012-2016	*n*= 82[c]	ST11 (17)	Mainly Germany	Uncertain	Dog	Carbapenemase-producers
			ST15 (61)	Mainly Germany	Uncertain	Cat and dog	Carbapenemase-producers
			ST307 (2)	Mainly Germany	Uncertain	Dog	Carbapenemase-producers
			ST661 (1)	Mainly Germany	Uncertain	Cat	Carbapenemase-producers
			ST895 (1)	Mainly Germany	Uncertain	Dog	Carbapenemase-producers
Chatzopoulou et al., 2018		*n*= 1	ST194 (1)	Greece	Urine (1)	Dog	ESBL-producers
Silva et al., 2018		*n*= 1	ST231 (1)	Brasil	Nasal discharge	Dog	ESBL-producers

Legend: [a]Total number of *K. pneumoniae* typed; [b]Remaining data from Harada et al. (2016); [c]5 strains included from Stolle et al. (2013).

The Institut Pasteur Bigsdb (2018) database includes additional STs isolated from animals, of which ST74, ST30, ST989 and ST1463 were obtained from companion animals with cystitis/UTI in Europe.

In a recent review, nine worldwide disseminated XDR *K. pneumoniae* high-risk clonal lineages[9] causing nosocomial outbreaks in humans were identified (Navon-Venezia et al., 2017). These XDR high-risk clonal lineages included ST11, ST14, ST15, ST17, ST37, ST101, ST147, ST258 and ST512 (Navon-Venezia et al., 2017). Other STs were acknowledged as important epidemic international outbreak clones (ST16, ST20, ST48, ST307, ST340, ST336 and ST395) even though, currently, they did not meet the criteria to be classified as high-risk clonal lineages (Navon-Venezia et al., 2017). Clonal group (CG) 258, which includes ST258, ST11 and ST512, were responsible for 68% of published nosocomial outbreaks followed by CG15 (~20%, includes ST15 and ST14) (Navon-Venezia et al., 2017). *K. pneumoniae* CG258, ST14, ST37 and ST147 are frequently carbapenemases-producers, while ST15 and ST17 are mostly ESBL-producers (Navon-Venezia et al., 2017). In parallel, important clonal lineages of hypervirulent *K. pneumoniae* such as ST23 have also been identified (Bialek-Davenet et al., 2014).

As in companion animals, studies determining the clonal lineages of *K. pneumoniae* isolated from humans usually focus on specific populations (e.g. carbapenemase-producers) or nosocomial outbreaks, thus, not aiming at characterising the specific epidemiology in UTI.

Studies on *K. pneumoniae* clonal lineages in Portugal are lacking in companion animals and were mostly centred on ESBL/carbapenemase-producers from hospitals in humans (Table 7). The most frequent *K. pneumoniae* clonal lineages detected in urine samples from Portugal were the ST11, ST14, ST15, ST147, ST348 and ST336 (Table 7).

1.4.1.4 Ribosomal multilocus sequence typing

The comparison of the ribosomal RNA gene restriction patterns is the bases for the rybotyping method, extensively used in *K. pneumoniae* (Brisse et al., 2006). The 16S ribosomal RNA (16S rRNA) genes are present in all bacteria, thus providing a general framework for analysis of bacterial diversity and classification (Jolley et al., 2012). With the expansion of whole genome sequence methodologies, the 16S rRNA genes became more easily accessible. Jolley et al. (2012) proposed a new MLST scheme, the ribosomal MLST (rMLST), that compares the 53 loci of 16S rRNA and can be used regardless of the bacterial species being studied. Since rMLST includes a larger number of loci that are shared by all bacteria, it is more discriminatory than MLST and can be used as a universal typing tool (Jolley et al., 2012). Nevertheless, rMLST does not preclude the usefulness of MLST for epidemiological purposes (Jolley et al.,

[9] High-risk clonal lineages were defined as clones that caused at least four recognised outbreaks and that were reported from ≥10 countries (Navon-Venezia et al., 2017).

Table 7 - Sequence types described in *K. pneumoniae* from humans from Portugal

Study	Year	Total number[a]	Clones (number)	Source (number)	Origin	Choice reasoning
Diancourt et al., 2005	Unknown	*n*= 2	ST12 (1)	Blood	Hospital	-
			ST15 (1)	Urine (1)	Hospital	-
Machado et al., 2010	2010	*n*= 2	ST11 (2)	Blood	Hospital	Carbapenemase-producers
Calisto et al., 2012	2009-2011	*n*= 4	ST14 (4)	Uncertain	Hospital	Carbapenem-resistant
Novais et al., 2012	2010	*n*= 10	ST14 (1)	Urine (1)	Hospital	Ertapenem-resistant
			ST15 (8)	Urine (5) and blood	Hospital	Ertapenem-resistant
			ST45 (1)	Exudate	Hospital	Ertapenem-resistant
Rodrigues et al., 2014a[b]	2006-7/2010	*n*= 57	ST14 (1)	Exudate	Hospital	ESBL-producers
			ST15 (16)	Urine (12), spuctum and other	Hospital	ESBL-producers
			ST147 (9)	Urine (5), blood and other	Hospital	ESBL-producers
			ST336 (31)	Urine (27), blood and exudate	Hospital	ESBL-producers
Rodrigues et al., 2014b	2011-2012	*n*= 2	ST15 (2)	Urine (2)	Hospital	Carbapenemase-producers
Manageiro et al., 2015	2006-2013	*n*= 26	ST11 (2)	Uncertain	Hospital	Carbapenemase-producers
			ST14 (3)	Uncertain	Hospital	Carbapenemase-producers
			ST15 (4)	Uncertain	Hospital	Carbapenemase-producers
			ST34 (1)	Uncertain	Hospital	Carbapenemase-producers
			ST59 (1)	Uncertain	Hospital	Carbapenemase-producers
			ST147 (4)	Uncertain	Hospital	Carbapenemase-producers
			ST231 (4)	Uncertain	Hospital	Carbapenemase-producers
			ST416 (1)	Uncertain	Hospital	Carbapenemase-producers
			ST698 (1)	Uncertain	Hospital	Carbapenemase-producers
			ST960 (1)	Uncertain	Hospital	Carbapenemase-producers

Table 7 (continuation)- Sequence types described in *K. pneumoniae* from humans from Portugal

Study	Year	Total number[a]	Clones (number)	Source (number)	Origin	Choice reasoning
Manageiro et al., 2015			ST1138 (4)	Uncertain	Hospital	Carbapenemase-producers
Papagiannitis et al., 2015	2009	*n*= 1	ST252 (1)	Faecal carriage	Hospital	ESBL/carbapen.-producers
Rodrigues et al., 2016	2014-2015	*n*= 20	ST15 (5)	Urine (4) and spuctum	Community[c]	Carbapenemase-producers
			ST109 (1)	Urine (1)	Community[c]	Carbapenemase-producers
			ST147 (10)	Urine (10)	Community[c]	Carbapenemase-producers
			ST231 (3)	Urine (3)	Community[c]	Carbapenemase-producers
			ST348 (1)	Urine (1)	Community[c]	Carbapenemase-producers
Vubil et al., 2017	2013	*n*= 6	ST15 (3)	Urine (2) and sputum	Hospital	Ertapenem-resistant
			ST348 (2)	Urine (1) and sputum	Hospital	Ertapenem-resistant
			ST11 (1)	Urine (1)	Hospital	Ertapenem-resistant
Rodrigues et al., 2017	2015-2016	*n*= 16	ST15 (11)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
			ST11 (1)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
			ST348 (1)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
			ST262 (1)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
			ST252 (1)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
			ST661 (1)	Faecal carriage	Long-term care facility	ESBL/carbapen.-producers
Bandeira et al., 2017	1980/2010	*n*= 3	ST14 (2)	Skin and surgical wound	Health-care-associated	-
			ST15 (1)	Urine (1)	Health-care-associated	-
Pereira et al., 2017	-	*n*= 1	ST23 (1)	Hepatic abscess	Community	-
Mendes et al., 2018	2015-2017	*n*= 24	ST45 (23)	Urine (3), faecal carriage, blood and peritoneal fluid	Hospital	Carbapenemase and mcr-1
			ST1112 (1)	Pus	Hospital	Carbapenemase and mcr-1

Table 7 (continuation)- Sequence types described in *K. pneumoniae* from humans from Portugal

Study	Year	Total number[a]	Clones (number)	Source (number)	Origin	Choice reasoning
Manageiro et al., 2018	2013-2014	*n*= 13	ST11 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST14 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST15 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST17 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST34 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST45 (2)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST231 (2)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST348 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST395 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST1513 (2)	Uncertain	Hospital	ESBL/carbapen.-producers
Caneiras et al., 2018	1990-2011	*n*= 25	ST11 (2)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST14 (15)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST15 (5)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST133 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST 147 (1)	Uncertain	Hospital	ESBL/carbapen.-producers
			ST276 (1)	Uncertain	Hospital	ESBL/carbapen.-producers

Legend: [a]Total number of *K. pneumoniae* typed; [b]Includes reference to unpublished data stating that the *K. pneumoniae* ST15 is common in community settings in Portugal; [c]Long-term care facilities, nursing homes and ambulatory.

2012). As with the MLST scheme, the rMLST alleles are freely available on the BigsDB database (Institut Pasteur Bigsdb, 2018).

1.4.1.5 Core multilocus sequence typing

The core multilocus sequence typing (cgMLST) comes as a natural evolution of the use of next generation sequence technologies aiming at higher discriminatory power and a more precise definition of relevant resistant and/or virulent clonal groups (Bialek-Davenet et al., 2014). In Bialek-Davenet et al. (2014) cgMLST scheme, a set of 634 core *loci*, defined as strict cgMLST, are combined with the 7 MLST genes and 53 rMLST genes. The cgMLST was shown to be more discriminatory, reproducible and to overcome spurious ST associations obtained by MLST analysis (Bialek-Davenet et al., 2014). So far, cgMLST showed that, overall, MDR CGs do not seem to overlap the hypervirulent *K. pneumoniae* CGs (Bialek-Davenet et al., 2014). Although cgMLST is more discriminatory, studies on recent outbreaks may require more resolution by using additional loci (Bialek-Davenet et al., 2014).

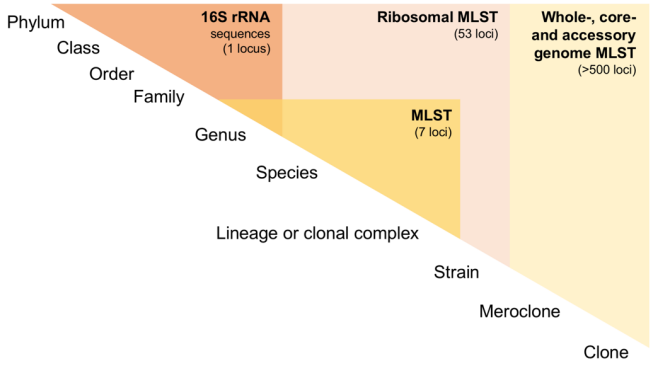
1.4.1.6 Whole genome sequencing

Whole genome sequencing (WGS) is a powerful tool that provides access to most of the bacterial genome sequence (Maiden et al., 2013; Quainno et al., 2017). Therefore, WGS provides maximal strains discrimination for epidemiological typing (Struelens & Brisse, 2013; Quainno et al., 2017). On-line databases are available, including for *K. pneumoniae*, that allow easy retrieval of typing information such as MLST, rMLST or cgMLST as well as data on resistance and virulence determinants (Maiden et al., 2013; Struelens & Brisse, 2013; Quainno et al., 2017; Institut Pasteur Bigsdb, 2018). Methods to compare WGS data include single nucleotide polymorphisms (SNP) and whole-genome MLST (wgMLST) analysis (Maiden et al., 2013; Quainno et al., 2017). The comparison of a larger amount of the bacterial genome allows for higher resolution between isolates and is useful for epidemiological purposes such as the characterisation of nosocomial outbreaks (Figure 6) (Quainno et al., 2017). Currently, WGS is considered the ultimate tool since it is portable, reproducible and allows maximum resolution (Struelens & Brisse, 2013; Quainno et al., 2017).

1.4.2 Antimicrobial resistance genes

Besides being an important pathogen, *K. pneumoniae* is also a major source of antimicrobial resistance, which has increased in the past decades (Navon-Venezia et al., 2017; Martin & Bachman, 2018). *K. pneumoniae* shows higher resistance rates than *E. coli* when considering invasive isolates from Europe, highlighting their role in the worldwide antimicrobial resistance burden (Navon-Venezia et al., 2017).

Figure 6 – Molecular typing methods and discriminatory power (adapted from Maiden et al., 2013)



1.4.2.1 β-Lactams

K. pneumoniae are intrinsically resistant to ampicillin, amoxicillin, carbenicillin and ticarcillin due to constitutive expression of chromosomal encoded β-lactamases, namely from SHV family (Babini & Livermore, 2000; Hæggman, Löfdahl, Paauw, Verhoef & Brisse, 2004). Nevertheless, ampicillin-susceptible *K. pneumoniae* have been reported (Kahlmeter & ECO.SENS., 2003; Mendonça, Ferreira, Louro, ARSIP Participants & Caniça, 2009), which may be related to changes in SHV expression (Fu et al., 2007).

The first ESBL-producing *K. pneumoniae* were described in the 1980s and since then *K. pneumoniae* became a major ESBL-carrying pathogen, especially in hospital settings (Paterson & Bonomo, 2005; Reinert et al., 2007; Calbo & Garau, 2015). Although SHV and TEM ESBLs predominated initially, since the 2000s, CTX-M β-lactamases, particularly CTX-M-15, became dominant (Coque et al., 2008; Rossolini, D'Andrea & Mugnaioli, 2008; Calbo & Garau, 2015; Navon-Venezia et al., 2017). The worldwide dissemination of CTX-M-15 among *K. pneumoniae* seems to be a joint result of the dissemination of epidemic clonal lineages and the dissemination of different multidrug-resistant plasmids (Calbo & Garau, 2015). For instance, the high-risk clonal lineage ST15 seems to be mainly a CTX-M-15-producer (Navon-Venezia et al., 2017). Notably, the expansion of CTX-M-15-producing epidemic *K. pneumoniae* clones showing co-resistance to ciprofloxacin has been reported in Hungary (Damjanova et al., 2008). Nevertheless, *K. pneumoniae* has been found to harbour several ESBL enzymes, sometimes simultaneously (Machado et al., 2007; Navon-Venezia et al., 2017).

In companion animals, CTX-M-15 is among the most common ESBLs (frequencies as high as 85% of third-generation cephalosporin resistant *K. pneumoniae* in some studies), although other ESBLs have been also detected including SHV-2, -12, -27, TEM-52 and CTX-M-1, -2, -3, -9, -11, -14, -27, -55 (Haenni et al., 2012; Poirel et al., 2013; Stolle et al., 2013; Donati et al., 2014; Ewers et al., 2014b; Schmiedel et al., 2014; Wohlwend et al., 2015; González-Torralba et al., 2016; Harada et al., 2016; Kuan, Chang, Lee & Yeh, 2016; Ovejero et al., 2017;

Xia et al., 2017; Chatzopoulou et al., 2018; Maeyama et al., 2018; Pulss et al., 2018; Silva et al., 2018; Zhang et al., 2018). The high-risk clonal lineage ST15 harbouring CTX-M-15 seems to be disseminated in companion animals from several countries (Haenni et al., 2012; Stolle et al., 2013; Ewers et al., 2014b; Harada et al., 2016; Pulss et al., 2018). The co-production of CTX-M-15 with OXA-1 and/or TEM-1 has been repeatedly reported (Machado et al., 2006; Haenni et al., 2012; Poirel et al., 2013; Stolle et al., 2013; Donati et al., 2014; Kuan et al., 2016; Pulss et al., 2018).

Portugal has a high incidence of ESBL according to EARS-Net report and *K. pneumoniae* is among the most frequent ESBL-producer detected in humans (Machado et al., 2007; Mendonça et al., 2009; Manageiro et al., 2010). Furthermore, ESBL-producing *K. pneumoniae* frequently show co-resistance to other antimicrobial classes (Espinar, Rocha, Ribeiro, Gonçalves Rodrigues & Pina-Vaz, 2011). Studies of *K. pneumoniae* from Portuguese patients revealed the detection of TEM-3, -4, -10, -12, -24, -52; SHV-2, -5, -12, -55, -90, -106; GES-1, -7 and CTX-M-1, -3, -9, -15, -32, -61 ESBLs (Barroso et al., 2000; Duarte et al., 2003; Conceição, Brízio & Duarte, 2005; Machado et al., 2006; Mendonça, Ferreira & Caniça, 2006; Machado et al., 2007; Fernandes, Vieira, Ferraz & Prudêncio, 2008; Mendonça et al., 2009; Fernandes & Prudêncio, 2010; Ferreita et al., 2010; Novais et al., 2010; Calisto et al., 2012; Manageiro et al., 2012a; Manageiro et al., 2012b; Novais et al., 2012; Calhau, Boaventura, Ribeiro, Mendonça & da Silva, 2014; Fernandes, Amador, Oliveira & Prudêncio, 2014; Freitas, Machado, Ribeiro, Novais & Peixe, 2014; Rodrigues, Machado, Ramos, Peixe & Novais, 2014a; Rodrigues et al., 2016; Rodrigues et al., 2017; Caneiras et al., 2018; Manageiro et al., 2018). Additionally, several other non-ESBL SHV and TEM β-lactamases have also been described in Portugal (Mendonça et al., 2009; Calisto et al., 2012; Caneiras et al., 2018; Manageiro et al., 2018). The presence of ESBL in human patients from Portugal has been found mostly in ST15 but also in other epidemic clonal lineages such as ST11, ST147 and ST336 (Novais et al., 2012; Rodrigues et al., 2014a; Managueiro et al., 2015; Rodrigues et al., 2016; Rodrigues et al., 2017).

Although less frequently than ESBLs, *K. pneumoniae* may also harbour several plasmid-mediated AmpC β-lactamases (Jacoby, 2009). DHA-1 followed by CMY-2 are the most common AmpC β-lactamases detected in *K. pneumoniae* from companion animals (Ma et al., 2009; Hidalgo et al., 2013; Stolle et al., 2013; Poirel et al., 2013; Bogaerts et al., 2014; Donati et al., 2014; Wohlwend et al., 2015; González-Torralba et al., 2016; Harada et al., 2016; Liu, Yang, Chen & Xia, 2017; Ovejero et al., 2017; Taniguchi et al., 2017; Maeyama et al., 2018; Pulss et al., 2018). CMY-2 and DHA-1 are also the most common AmpC β-lactamases in *K. pneumoniae* from humans (Freitas et al., 2014; Navon-Venezia et al., 2017), and the latter has been detected in several studies in Portugal (Manageiro et al., 2012b; Ferreira et al., 2010; Freitas et al., 2014; Rodrigues et al., 2017; Vubil et al., 2017; Manageiro et al., 2018). Third-generation cephalosporin-resistance in *K. pneumoniae* may also be related with the

combination of SHV-1 hyperproduction and outer membrane modifications (Rice et al., 2000). Furthermore, the combination of ESBL/AmpC β-lactamases with porin loss (e.g. OmpK36 and OmpK35) or increased efflux in *K. pneumoniae* may lead to carbapenem-resistance (Bradford et al., 1997; Calisto et al., 2012; Navon-Venezia et al., 2017; Hamzaoui et al., 2018).

K. pneumoniae is a major carbapenemase-producing pathogen that may harbour a wide range of carbapenemases (Queenan & Bush, 2007; Albiger et al., 2015; Grundmann et al., 2017; Navon-Venezia et al., 2017). CG258, ST14, ST37, ST147 and ST101 are the clonal lineages more frequently found associated with carbapenemases worldwide (Navon-Venezia et al., 2017). In Europe, KPC followed by OXA-48-like are the most disseminated carbapenemases in *K. pneumoniae* overall (Grundmann et al., 2017). In Portugal, KPC-3 was found to be the most common KPC among *K. pneumoniae* and was frequently associated with high-risk clonal lineages such as ST11, ST14, ST15 and ST147 (Manageiro et al., 2015; Rodrigues et al., 2016; Vubil et al., 2017; Caneiras et al., 2018; Manageiro et al., 2018). In companion animals, there are only sporadic reports of the detection of carbapenemase-producing *K. pneumoniae* harbouring OXA-48 in Spain and Germany (Stolle et al., 2013; Schmiedel et al., 2014; González-Torralba et al., 2016; Pulss et al., 2018).

1.4.2.2 Fluoroquinolones

K. pneumoniae may harbour all the known quinolone-resistance mechanisms described in Gram-negative bacteria (Navon-Venezia et al., 2017).

K. pneumoniae from companion animals have been found to frequently have *gyrA* and *parC* mutations (Guillard et al., 2016; de Jong et al., 2018). Although, less frequently, *aac(6')-Ib-cr, qnrB*, *qnrS* and *qnrA* have also been detected (Hidalgo et al., 2013; Stolle te al., 2013; Ewers et al., 2014b; Donati et al., 2014; Schlüter et al., 2014; Schmiedel et al., 2014; Wohlwend et al., 2015; Harada et al., 2016; Guillard et al., 2016; Ovejero et al., 2017; Chatzopoulou et al., 2018; de Jong et al., 2018; Silva et al., 2018; Zhang et al., 2018). In *K. pneumoniae* from companion animals, *aac(6')-Ib-cr* followed by *qnrB* seems to be the more common PMQR, in particular among ESBL-producers (Ma et al., 2009; Stolle te al., 2013; Donati et al., 2014; Wohlwend et al., 2015; Harada et al., 2016; de Jong et al., 2018). Furthermore, chromosome encoded OqxAB is highly frequent in *K. pneumoniae* from companion animals while QepA is rare (Ma et al., 2009; Harada et al., 2016; Guillard et al., 2016; de Jong et al., 2018).

K. pneumoniae isolated from humans, including in Portugal, show similar resistant determinants to quinolones as those described in companion animals (Deguchi et al., 1997; Machado et al., 2006; Ferreira et al., 2010; Rodríguez-Martínez et al., 2011; Ruiz et al., 2012; Rodríguez-Martínez et al., 2013; Jacoby et al., 2014; Schmiedel et al., 2014; Holt et al., 2015; Wohlwend et al., 2015; Navon-Venezia et al., 2017; Mendes et al., 2018). Adicionally, *qnrD* has been seldomly detected in human *K. pneumoniae* (Zhang et al., 2013).

1.4.2.3 Other antimicrobials

Most studies on resistance determinants of *K. pneumoniae* epidemiology are focussed on β-lactamases (ESBLs, AmpCs and carbapenemases-producers) and on fluoroquinolones to a lesser extent. Also, there is only a limited number of studies including *K. pneumoniae* from companion animals. Nevertheless, *K. pneumoniae* may acquire resistance determinants to several other antimicrobial classes (Wyres & Holt, 2016; Holt et al., 2015).

Notably, Holt et al. (2015) detected more than 100 resistance determinants in a large collection of *K. pneumoniae* from humans and bovines by WGS. In addition to *oqxAB* and bla_{SHV}, *fosA*, which confers low resistance to fosfomycin, was found as part of *K. pneumoniae* core resistome (Holt et al., 2015).

In Holt et al. (2015) study, *sul1* and *sul2* frequency in *K. pneumoniae* was high while *sul3* was rarely found. In *K. pneumoniae* from companion animals, the same seems to be true (Donati et al., 2014; Ewers et al., 2014b; Schlüter et al., 2014; Wohlwend et al., 2015; González-Torralba et al., 2016; Zhang et al., 2018). Nevertheless, *sul1* and *sul2* frequencies vary between studies (Donati et al., 2014; Ewers et al., 2014b; Zhang et al., 2018). Several *dfrA* genes have been reported in *K. pneumoniae* from humans and companion animals, with *dfrA14* and *dfrA12* being the most common in most studies (Donati et al., 2014; Schlüter et al., 2014; Holt et al., 2015; Wohlwend et al., 2015; González-Torralba et al., 2016; Taitt et al., 2017; Silva et al., 2018; Zhang et al., 2018).

Regarding aminoglycosides, Holt et al. (2015) also detected several AMEs genes, including *aac(3)-II* and *aac(6')-Ib-cr*, that showed the higher frequencies. The *acc(3)-II* and *acc(6')-Ib* were also the main detected genes by Haldorsen, Simonsen, Sundsfjord and Samuelsen (2014) in *K. pneumoniae* from Norway. Interestingly, Taitt et al. (2017) found lower *aac(6')-Ib* frequencies in faecal *K. pneumoniae* from humans from Kenya. As mentioned above, *aac(6')-Ib-cr* is common among *K. pneumoniae* from companion animals while little in known about other AMEs (Ma et al., 2009; Stolle te al., 2013; Donati et al., 2014; Wohlwend et al., 2015; Harada et al., 2016; de Jong et al., 2018). Plasmid-mediated 16S rRNA methylases have been detected in *K. pneumoniae* from animals and humans, although reports from companion animals are scarse (Deng et al., 2011; Wachino & Arakawa, 2012; Hidalgo et al., 2013; Xia et al., 2017).

K. pneumoniae may harbour a wide number of different tetracycline-resistance *tet* genes (Roberts, 2017). According to Holt et al. (2015) findings, *tet(A)* was the most disseminated followed by *tet(D)*. Taitt et al. (2017) also frequently found *tet(B)* in *K. pneumoniae* from Kenya. In companion animals, Ewers et al. (2014b) detected *tet(A)* in most *K. pneumoniae* (~50%) while *tet(B)* and *tet(C)* were less common. Furthermore, Donati et al. (2014) and Chatzopoulou et al. (2018) also found *tet(D)* in *K. pneumoniae* from companion animal.

Finally, Holt et al., 2015 study showed the presence of *catA*, *catB*, *cmlA* and *floR* chloramphenicol-resistance genes in *K. pneumoniae* from humans and bovine. The *catA*, *catB*

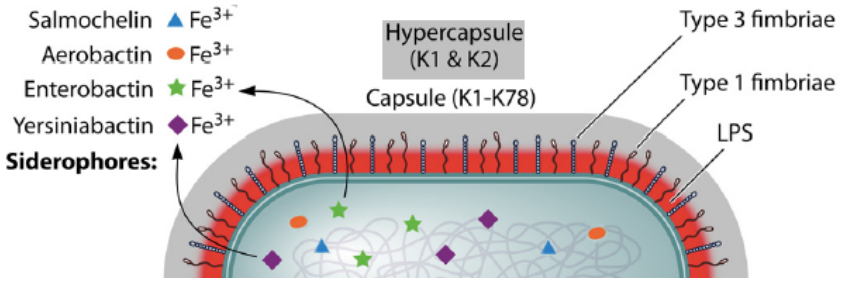
and *floR* have also been reported among *K. pneumoniae* from companion animals (Wohlwend et al., 2015; González-Torralba et al., 2016; Chatzopoulou et al., 2018; Silva et al., 2018; Zhang et al., 2018).

1.4.3 Virulence

K. pneumoniae virulence factors are encoded in its core genome but also in accessory genomes such as plasmids (Martin & Bachman, 2018). *K. pneumoniae* is highly resilient due to its defensive rather than offensive mechanisms (Paczosa & Mecsas, 2016). Nevertheless, the emergence of hypervirulent *K. pneumoniae* (capable of causing disease in otherwise healthy patients), as opposed to classic *K. pneumoniae*, is worrisome (Shon, Bajwa & Russo, 2013; Paczosa & Mecsas, 2016). Currently, MDR *K. pneumoniae* seems to be associated with less virulent strains and vice versa (Bialek-Davenet et al., 2014; Paczosa & Mecsas, 2016). However, there are already some reports of resistant and hypervirulent *K. pneumoniae* strains (Bialek-Davenet et al., 2014; Holt et al., 2015; Paczosa & Mecsas, 2016; Harada & Doi, 2018). Studies reporting the virulence of *K. pneumoniae* from companion animals are rare (Sato et al., 2018).

K. pneumoniae has four main virulence factor groups that have been extensively studied, namely the capsule, the lipopolysaccharide (LPS) and several siderophores and fimbriae (Paczosa & Mecsas, 2016) (Figure 7).

Figure 7 – *K. pneumoniae* main virulence factors (adapted from Paczosa and Mecsas, 2016)



1.4.3.1 Capsule

The capsule is a complex acid polysaccharide matrix that is attached to the outside of the *K. pneumoniae* outer membrane (Podschun & Ullmann, 1998). The capsule is a major virulence factor used by *K. pneumoniae* to evade the immune system during infection through multiple mechanisms (Paczosa & Mecsas, 2016). Notably, acapsular *K. pneumoniae* have been shown to be less virulent including in *in vivo* UTI models (Camprubí, Merino, Benedí & Tomás, 1993; Struve & Krogfelt, 2003; Paczosa & Mecsas, 2016). As mentioned before, the capsule may be used for *K. pneumoniae* typing by serological or molecular methods. Capsule types K1 and K2 seems to be more virulent and also highly associated with hypervirulent

K. pneumoniae (Paczosa & Mecsas, 2016; Martin & Bachman, 2018). Furthermore, some serotypes, seem to be more frequent in clinical isolates, with K2 being common among classic *K. pneumoniae* strains causing UTI (Riser & Noone, 1981; Podschun, Sievers, Fischer & Ullmann, 1993; Lin et al., 2014; Paczosa & Mecsas, 2016).

K. pneumoniae may have a hypermucoviscous phenotype, associated with the capsule hyper-production, that is characterised by a positive "string" test[10], which may contribute to a higher *K. pneumoniae* virulence (Shon et al., 2013; Paczosa & Mecsas, 2016; Martin & Bachman, 2018). This hypermucoviscous phenotype is frequently associated with hypervirulent *K. pneumoniae* strains, although it is important to notice that it has also been detected in classic *K. pneumoniae* (Catalán-Nájera, Garza-Ramos & Barrios-Camacho, 2017). The hypermucoviscous phenotype may result from the expression of the regulator of mucoid phenotype genes *rmpA* and *rpmA2*; or with the regulation of the capsule synthesis A and B genes (*rcsA* and *rcsB*) (Shon et al., 2013; Paczosa & Mecsas, 2016). Moreover, the hypermucoviscous phenotype may be associated with the chromosomal-associated gene A (*magA*), which was found to be specific of K1 capsule type and thus later renamed *wzy*_K1 (Paczosa & Mecsas, 2016). External cues such as increased glucose or iron concentrations may also increase or decrease capsule production, respectively (Paczosa & Mecsas, 2016). Notably, the detection of conflicting phenotypes/genotype associations, such as *rpmA*/*A2*-positive strains without hypermucoviscous phenotype highlights the need for further studies to uncover the complexity of this *K. pneumoniae* phenotype (Catalán-Nájera et al., 2017). A human infection caused by a hypervirulent and hypermucoviscous *K. pneumoniae* has been recently reported in Portugal, although its corresponding genotype was not determined (Pereira et al., 2017).

1.4.3.2 Lipopolysaccharide

The LPS is a component of the Gram-negative cell membrane and is typically composed of an O-antigen, a core oligosaccharide, and lipid A (Paczosa & Mecsas, 2016). *K. pneumoniae* lacking or producing incomplete LPS seem to be less virulent (Paczosa & Mecsas, 2016). LPS protects the bacteria against humoral immune response but may also act as an immune activator (Paczosa & Mecsas, 2016). For instance, LPS protects bacteria against the complement by means of the O-antigen (Li, Zhao, Liu, Chen & Zhou, 2014). Furthermore, O-antigens have been implicated in increased serum-resistance together with the capsule and other membrane lipoproteins, such as the TraT lipoprotein (Podschun & Ullmann, 1998). It shoud be noted that some studies point out that LPS and serum-resistance may be important virulent factors for UTI (Camprubí et al., 1993; Podschun et al., 1993).

[10]Positive "string test" means that touching a colony with a loop results in a strand >5-10mm (Shon et al., 2013; Martin & Bachman, 2018).

1.4.3.3 Siderophores and iron transport systems

Iron, which is essential for bacterial survival, is not readily available in most infection sites such the urinary tract (Podschun & Ullmann, 1998; Paczosa & Mecsas, 2016). Siderophores are low-molecular weight molecules with high affinity to iron that are secreated by bacteria to increase iron acquisition and are, therefore, important virulence factors (Holden & Bachman, 2015; Paczosa & Mecsas, 2016). *K. pneumoniae* have been shown to secrete several types of siderophores with different virulence capabilities and iron affinities (Holden & Bachman, 2015; Paczosa & Mecsas, 2016). Siderophore systems may be dependent on TonB which is encoded in *K. pneumoniae* core genome, regulates iron transport and acts as a virulence factor (Martin & Bachman, 2018).

Enterobactin is a catecholate siderophore that is part of the core genome of *K. pneumoniae* and thus is ubiquitous and a primary iron uptake sytem (Podschun & Ullmann, 1998; Paczosa & Mecsas, 2016; Martin & Bachman, 2018). However, the hosts innate immune system has developed conterating ways, namely through the action of lipocain-2 (Holden & Bachman, 2015; Paczosa & Mecsas, 2016; Martin & Bachman, 2018). Enterobactin biosynthesis and transport mediating proteins are chromosome encoded in the *entABCDEF* and *fepABCDG* gene clusters, respectively (Paczosa & Mecsas, 2016). Salmochelin is a c-glucosylated enterobactin-derivative catecholate siderophore which is able to evade the action of lipocain-2, consequently leading to higher virulence (Holden & Bachman, 2015; Paczosa & Mecsas, 2016). Salmochelin codifying genes (*iroABCDE* gene cluster) may be chromosomal or plasmid encoded (Paczosa & Mecsas, 2016). The membrane receptor of salmochelin (IroN) also has specificity for other siderophores and has been shown to contribute to the ivasion of urothelial cells by *E. coli in vitro* (Feldmann, Sorsa, Hildinger & Schubert, 2007; Müller, Valdebenito & Hantke, 2009). Interestingly, salmochelin and IroN prevalence has been shown to be higher in hypervirulent *K. pneumoniae* strains (Hsieh, Lin, Lee, Tsai & Wang, 2008; Holt et al., 2015).

Yersiniabactin is a mixed-type siderophore that was originally identified in *Yersinia* species and is encoded in a chromosomal “high pathogenicity island” containing genes for its biosynthesis, transport, and regulation (Paczosa & Mecsas, 2016; Martin & Bachman, 2018). It may have additional functions such as copper-resistance (Holden & Bachman, 2015; El Fertas-Aissani, Messai, Alouache & Bakour, 2013). Yersiniabactin can evade lipocain-2 (Bachman et al., 2011) and has been linked to invasive *K. pneumoniae* infections (Holt et al., 2015). In fact, yersiniabactin is particularly frequent among hypervirulent *K. pneumoniae* in contrast to classic *K. pneumoniae* (~17% overall and 9% in urine samples) (Hsieh et al., 2008; Bachman et al., 2011; Compain et al., 2014). Nevertheless, El Fertas-Aissani et al. (2013) detected higuer frequencies also in isolates from UTI (~30%).

Aerobactin, is also a mixed-type siderophore that is usually plasmid-encoded (Martin & Bachman, 2018). Aerobactin is a major virulence factor in *K. pneumoniae* since it is linked to

higher invasiveness and is extremely common among hypervitulent *K. pneumoniae* (Hsieh et al., 2008; Compain et al., 2014; Holt et al., 2015; Paczosa & Mecsas, 2016). On the other hand, it is less frequent (7-18%) among classic *K. pneumoniae* (Russo et al., 2014). Aerobaction is usually associated with *rpmA* since aerobactin codifing gene cluster (*iucABCD*) and transporter (*iutA*) are frequently encoded in the same plasmid (Russo et al., 2014; Holt et al., 2015; Paczosa & Mecsas, 2016).

Finally, Kfu is an ABC iron transport system that seem to be associated to hipervirulent *K. pneumoniae* strains (Hsieh et al., 2008; Compain et al., 2014; Paczosa & Mecsas, 2016). Nonetheless, it is also present in classic *K. pneumoniae* (Holt et al., 2015; Sato et al., 2018).

The secretion of several sidephores may lead to better bacterial evation to the hosts immune system and may lead to increased collonization success (Koczura & Kaznowski, 2003; Russo et al., 2014; Holden & Bachman, 2015; Holt et al., 2015; Paczosa & Mecsas, 2016). Furthermore, siderophore specificities may impact the bacterial virulence (Holden & Bachman, 2015). In fact, studies in *E. coli* have shown that siderophores, particularly yersiniabactin and aerobactin, are important virulent factors for UTI (Garcia, Brumbaugh & Mobley, 2011). Hypervirulent *K. pneumoniae* usually harbour a higer number of siderophores that are also more active (Russo et al., 2011; Russo et al., 2014). Aerobactin seems to have a pivotal role in successful infection by hypervirulent *K. pneumoniae* over enterobactin, yersiniabactin or salmochelin (Russo et al., 2014; Russo, Olson, MacDonald, Beanan & Davidson, 2015).

In *K. pneumoniae* isolated from UTI, enterobactin is ubiquitous and followed in frequency by Kfu and yersiniabactin, while aerobactin and IroN are seldom detected (Tarkkanen et al., 1992; Podschun et al., 1993; Koczura & Kaznowski, 2003; El Fertas-Aissani et al., 2013; Calhau et al., 2014; Ranjbar, Memariani, Sorouri & Memariani, 2016; Sato et al., 2018). Interistingly, aerobactin was apparently more frequent among strains causing pyelonephritis when compared to cystitis or asymptomatic UTI in humans (Podschun et al., 1993).

1.4.3.4 Pilli (Fimbriae)

Pilli (fimbriae) are filamentous structures extending from the surface of bacteria that play an important role in bacterial adhesion to the host cells (Paczosa & Mecsas, 2016; Martin & Bachman, 2018). Two main types of pilli have been described in *K. pneumoniae,* namely type-1 and type-3 pilli (Martin & Bachman, 2018). These seem to be encoded in the *K. pneumoniae* core genome and are therefore highly frequent, including in strains causing UTI (Podschun & Sahly, 1991; Tarkkanen et al., 1992; Podschun et al., 1993; El Fertas-Aissani et al., 2013; Paczosa & Mecsas, 2016; Ranjbar et al., 2016; Sato et al., 2018; Martin & Bachman, 2018).

Although, other adhesive structers have been identified in *K. pneumoniae*, these have been scarselly characterised (Wu, Huang, Fung & Peng, 2010; Khater et al., 2015; Paczosa & Mecsas, 2016).

Type-1 pili are “mannose-sensitive”, thin and thread-like cell protrusions frequently expressed by *K. pneumoniae* that seem to be involved in adherence to human mucosal or epithelial surfaces (Paczosa & Mecsas, 2016). The *K. pneumoniae* and *E. coli* type-1 pilli gene cluster is homologous but different (Struve, Bojer & Krogfelt, 2008; Struve, Bojer & Krogfelt, 2009). The FimH minor subunit is located on the tip of type-1 pilli and is responsible for its adhesive properties (Paczosa & Mecsas, 2016). Type-1 pilli contributes to UTI development although it seems not to be involved in early collonization (Podschun et al., 1993; Rosen et al., 2008; Struve et al., 2008; Struve et al., 2009; Murphy, Mortensen, Krogfelt & Clegg, 2013). Interestingly, Stahlhut et al. (2009) found that FimH is targeted by adaptive point mutations, suggesting a pathoadaptive nature of this adhesin (Stahlhut et al., 2009).

Type-3 pili are “mannose-insensitive”, helix-like filaments which adhere to cell surfaces and are strong biofilm promoters (Struve et al., 2009; Schroll, Barken, Krogfelt & Struve, 2010; Paczosa & Mecsas, 2016). The *mrkD* gene included in the type-3 pilli gene cluster, encodes for the MrkD adhesin located in the pilli tip (Paczosa & Mecsas, 2016). Nevertheless, it is MrkA that seems to be the key for biofilm formation (Langstraat, Bohse & Clegg, 2001). Type-3 pilli have been shown to bing type IV and V collagen (Sebghati, Korhonen, Hornick & Clegg, 1998). Although type-3 pilli can bind to bladder epithelial cells *in vitro*, it does not seem to contribute to UTI (Struve et al., 2009; Paczosa & Mecsas, 2016). However, type-3 pilli may be a significant virulence factor in catheter associated UTIs (CAUTI) due to their ability to promote biofilm formation (Schroll et al., 2010).

1.4.3.5 Other virulence factors

Additional less characterised virulence factors have been identified in *K. pneumoniae*, such as the outer membrane protein porins, efflux pumps and genes involved in allantoin metabolism (Brisse et al., 2006; Li et al., 2014; Paczosa & Mecsas, 2016). The peptidoglycan-associated lipoprotein (Pal) and murein lipoprotein (LppA) are among the outer membrane proteins (OMP) that have been considered as important for *K. pneumoniae* virulence (Hsieh et al., 2013). Although the lack of OmpK36 porin has been associated with changes in antimicrobial resistance, its presence has been shown to contribute to *K. pneumoniae* virulence (Li et al., 2014; Paczosa & Mecsas, 2016).

Efflux pumps are also frequently associated with antibiotic resistance in *K. pneumoniae* due to their ability to export antibiotics from bacterial cells (Martin & Bachman, 2018). Some efflux pumps, such as AcrAB, also play a role in virulence in the lung (Padilla et al., 2010; Paczosa & Mecsas, 2016).

The allantoin utilisation system allows *K. pneumoniae* to obtain nitrogen and carbon from the environment and may be encoded in the core or accessory genome (Martin & Bachman, 2018). The presence of an allantoin utilisation operon has been associated with hypervirulent *K. pneumoniae* strains that cause pyogenic liver abscesses (Chou et al., 2004; Hsieh et al.,

2008; Compain et al., 2014). Furthermore, the deletion of a regulator gene (*allS*) in the allantoin utilisation system operon reduced *K. pneumoniae* virulence (Chou et al., 2004).

1.5 *Proteus mirabilis*

Proteus species are motile, Gram-negative bacteria that are well known for their swarming ability across agar surfaces (O'hara, Brenner & Miller, 2000; Szabo & Paterson, 2002; Drzewiecka, 2016). *Proteus* species can be found in the environment, food and colonising several animals including humans and companion animals (O'hara et al., 2000; Manos & Belas, 2006; Drzewiecka, 2016).

P. mirabilis is the most frequent *Proteus* species associated with human and companion animal infections (Szabo & Paterson, 2002; Manos & Belas, 2006; Drzewiecka, 2016). *P. mirabilis* is considered an opportunistic bacterium that is commonly isolated from uncomplicated, complicated and catheter associated UTIs (Szabo & Paterson, 2002; Manos & Belas, 2006; Drzewiecka, 2016). UTIs by *Proteus* species are linked with urinary stone formation, due to urine alkalinisation, and are prone to persistence and to secondary complications such as obstruction and pyelonephritis (Szabo & Paterson, 2002; Manos & Belas, 2006; Armbruster, Mobley & Pearson, 2018). In addition, *P. mirabilis* may be implicated in respiratory tract, eye, ear, nose, skin, throat, burns, wounds, among other infections (O'hara et al., 2000; Szabo & Paterson, 2002; Wang et al., 2014; Drzewiecka, 2016). Likewise, in companion animals, *P. mirabilis* is also a frequent cause of UTIs among other infections (Wooley & Blue, 1976; Normand et al., 2000a; Ling et al., 2001; Cohn et al., 2003; Harada et al., 2014; Schultz et al., 2015).

Although *P. mirabilis* is not included among ESKAPE nosocomial pathogens, it is frequently found as a nosocomial pathogen in humans especially when associated with CAUTI (Armbruster & Mobley, 2012; Armbruster et al., 2018). Furthermore, *P. mirabilis* may acquire several antimicrobial resistant mechanisms, such as ESBL, AmpC and Carbapenemase β-lactamases, and therefore also play an important role in antimicrobial resistance (Szabo & Paterson, 2002).

1.5.1 Population structure

Proteus species taxonomy has evolved over time (O'hara et al., 2000; Manos & Belas, 2006). *Proteus* species are traditionally considered as part of the Enterobacteriaceae family and the Proteeae tribe which also includes *Morganela* spp. and *Providencia* spp. (O'hara et al., 2000). More recently, Adeolu, Alnajar, Naushad and Gupta (2016) proposed the division of the Enterobacteriaceae family reassining the *Proteus* genus into a *Morganellaceae fam*. nov. based on WGS data of more than 1500 genes. First decribed in 1985, the genus *Proteus* currently includes several species with *P. mirabilis* being by far the most common in humans and companion animals (O'hara et al., 2000; Manos & Belas, 2006).

There are several typing systems that have been used for Proteeae characterisation such as phage, bacteriocin, protein profile, serological, restriction fragment length polymorphisms, PCR, PFGE and more recently WGS (O'hara et al., 2000; Manos & Belas, 2006; Aogáin, Rogers & Crowley, 2016). However, comparable typing systems worldwide such as MLST have not been established yet.

The Dienes test is a typing method based on the mutual inhibition of two non-isogenic *Proteus* spp. strains when swarming towards one another on an agar surface (Manos & Belas, 2006; Pfaller, Mujeeb, Hollis, Jones & Doern, 2000). When genetically different, a clear line will form on the edge of the swarming where the stains meet (Pfaller et al., 2000). Interestingly, Dienes test seems to have a high discriminatory power as other, more complex, typing methods such as ribotyping and PFGE (Pfaller et al., 2000). This test is not frequently used due to difficulties in result interpretation (Manos & Belas, 2006). Another important limitation is that it does not allow the determination of more complex phylogenetic relationships (Sabbuba, Mahenthiralingam & Stickler, 2003). Nevertheless, Dienes test has been used in epidemiological studies, one of which showed that there was no predominant *Proteus* spp. Dienes type among patients with bacteriuria (Clarke & McIntyre, 1996).

Proteus species can also be typed according to the O-antigen (Drzewiecka, 2016). Interestingly, a high number of O-antigens have been described and some seem to be more associated with clinical strains (Drzewiecka, 2016).

A comparison of several PCR-based typing methods has shown that the random amplified polymorphic DNA (RADP) analysis, the enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR and the BOX-PCR allowed a good discrimination among *P. mirabilis* clinical isolates and have the advantage of being faster than PFGE or ribotyping (Michelim et al., 2008). However, Michelim et al. (2008) did not compare the PCR-based results with PFGE. PFGE is still one of the gold standard methods for typing bacteria (Sabat et al., 2013). Like *K. pneumoniae*, there is no standard PFGE protocol proposed by PulseNet for *P. mirabilis* (CDC, 2016). Two main restriction enzymes are used by most authors, namely *Sfi*I and *Not*I (Sabbuba et al., 2003; Goering, 2010). By using PFGE, Sabbuba et al. (2003) highlighted the persistence of *P. mirabilis* causing UTI by confirming that a single genotype of *P. mirabilis* from catheterised patients can persist despite changes of catheter, periods of non-catheterisation and antibiotic therapy. To date, only two studies have included and compared strains from companion animals and humans by PFGE (Schultz et al., 2015; Schultz, Cloeckaert, Doublet, Madec & Haenni, 2017b). These studies included a small and selected resistant population of *P. mirabilis* from different infection sites. Notably, one of these studies found one close related strain infecting one unrelated companion animal and one human (Schultz et al., 2015).

The use of next-generation sequencing techniques provides a greater phylogenetic resolution and overcomes some limitations of 16S rRNA based studies (Choudoir, Campbell & Buckley, 2012). Studies using WGS of *P. mirabilis* are increasing, however there is still a lack of data

regarding companion animals. Reports on WGS of single or multiple *P. mirabilis* isolates are unravelling potential virulence genes, new resistance mechanisms and identifying potential multidrug-resistant clones (Pearson et al., 2008; Siebor & Neuwirth, 2014; Aogáin et al., 2016). A bigger number of studies using WGS (Aogáin et al., 2016) or the definition of a standardised MLST scheme are still needed for a better understanding of the population structure of *P. mirabilis* worldwide.

1.5.2 Antimicrobial resistance genes

P. mirabilis are naturally resistant to nitrofurantoin, tetracycline, tigecycline, polymixin B and colistin (Table 4) (Stock, 2003). In addition, *P. mirabilis* may present high minimum inhibitory concentrations to carbapenems, namely imipenem, through mechanism other than carbapenemases (Szabo & Paterson, 2002).

Some studies have shown that *P. mirabilis* resistance frequencies can be low (Fluit et al., 2000; Mutnick, Turner & Jones, 2002; Szabo & Paterson, 2002; Kahlmeter & ECO.SENS, 2003; Seguin et al., 2003; Authier et al., 2006; Pedersen et al., 2007; Harada et al., 2014; Kroemer et al., 2014; Wang et al., 2014; Criel et al., 2015; Moyaert et al., 2016). However, there are geographical variations and antimicrobial resistance frequencies may be higher according to the *P. mirabilis* source (Pedersen et al., 2007; Harada et al., 2014; Wang et al., 2014). For instance, ear isolates tend to be more resistant than *P. mirabilis* from UTIs in companion animals (Pedersen et al., 2007). Furthermore, some studies report the detection of important multidrug resistance genomic islands, namely *Salmonella* genomic island 1 (SGI1) and *Proteus* genomic island 1 (PGI1), in *P. mirabilis* from humans and companion animals highlighting its important role in the dissemination of resistance (Szabo & Paterson, 2002; Siebor & Neuwirth, 2014; Schultz et al., 2015). In fact, resistant and multidrug-resistant *P. mirabilis* is becoming more common in humans and companion animals (Normand et al., 2000a; Luzzaro et al., 2009).

1.5.2.1 β-Lactams

Proteus mirabilis is able to acquire plasmid and chromosomal β-lactamases including ESBLs, AmpCs and Carbapenemases (Chanal et al., 2000; Bonnet et al., 2002; Szabo & Paterson, 2002; Song et al., 2011; Schultz et al., 2017b). TEM-1 and TEM-2 are very common β-lactamases among amoxicillin-resistant *P. mirabilis* (~40%) from humans (Chanal et al., 2000; Harada et al., 2014; Huang et al., 2015). Several studies point to the emergence of ESBL and AmpC-producing *P. mirabilis* in humans (Luzzaro et al., 2009; Kanayama et al., 2010; D'Andrea et al., 2011; Song et al., 2011; Wang et al., 2014). Notably, some results also seem to point to the dissemination of specific ESBL or AmpC-producing *P. mirabilis* clones (Luzzaro et al., 2009; Kanayama et al., 2010; D'Andrea et al., 2011; Aogáin et al., 2016). CTX-M are among the most frequent ESBLs detected in *P. mirabilis*, but TEM followed by SHV ESBLs are

also found (Bonnet et al., 1999; Szabo & Paterson, 2002; Kanayama et al., 2010; Song et al., 2011; Huang et al., 2015). The *bla*$_{\text{CMY-2-like}}$ genes are the most common AmpCs detected in *P. mirabilis* from humans of several European countries (Aragón et al., 2008; D'Andrea et al., 2011; Song et al., 2011; Miró et al., 2013a). Interestingly, in Luzzaro et al. (2009) study, CMY-producers were mostly associated with UTIs. Chromosomal encoded CMY β-lactamase in *P. mirabilis* have been found as part of a SXT/R391-like integrative and conjugative element (ICE), which frequency seems to be increasing (Mata et al., 2011; Aogáin et al., 2016). In *P. mirabilis,* like in other Enterobacteriaceae, the ESBL/AmpC β-lactamases are frequently associated with resistance to other antimicrobials, such as fluoroquinolones (Kanayama et al., 2010; Wang et al., 2014; Aogáin et al., 2016).

Although less frequently than in other bacteria, carbapenemases are being increasingly reported in *P. mirabilis* from human patients leading to great therapeutic limitations. Several carbapenemases have been detected in *P. mirabilis*, including those belonging to OXA, NDM, IMP, VIM and KPC carbapenemase families (Bonnet et al., 2002; Szabo & Paterson, 2002; Miró et al., 2013a; Chen et al., 2015; Cabral, Maciel, Barros, Antunes & Lopes, 2015; Girlich, Dortet, Poirel & Nordmann, 2015; Di Pilato et al., 2016; Lange et al., 2017; Ohno et al., 2017; Potter et al., 2018; Valentin et al., 2018).

Studies on antimicrobial resistance mechanisms of *P. mirabilis* from humans and companion animals from Portugal are lacking. Nevertheless, one study on the genetic background of TEM-24 ESBL has included positive *P. mirabilis* strains from Portuguese patients (Novais et al., 2010). In companion animals, third-generation cephalosporin-resistant *P. mirabilis* frequently harbours CMY-2, which is worldwide disseminated (Dierikx et al., 2012; Hordijk et al., 2013; Harada et al., 2014; Schultz et al., 2017b). Other β-lactamases have also been described in companion animal *P. mirabilis*, namely PSE-1, OXA-10, CTX-M-2, -15, VEB-6, CMY-4 and DHA-1, -16 (Harada et al., 2014; Schultz et al., 2015; Schultz et al., 2017b; Maeyama et al., 2018; Zhang et al., 2018). Furthermore, TEM-1 is also common among *P. mirabilis* from companion animals (Harada et al., 2014).

1.5.2.2 Fluoroquinolones

P. mirabilis may acquire several fluoroquinolone-resistance mechanisms, with QRDR being more frequent than PMQR (Weigel et al., 2002; Jacoby et al., 2014). Interestingly, there are some QRDR mutations in *P. mirabilis* that are linked to higher resistance to enrofloxacin than to ciprofloxacin (Harada et al., 2014; Schultz et al., 2017a). Additionally, Weigel et al. (2002) found that *gyrB* mutations are surprisingly frequent among *P. mirabilis*.

P. mirabilis may harbour most *qnr* genes (Martínez-Martínez, Cano, Rodríguez-Martínez, Calvo & Pascual, 2008; Jacoby et al., 2014). Proteeae, which includes *P. mirabilis,* seems to be an essential carrier of *qnrD*-harbouring plasmids (Zhang et al., 2013; Hooper & Jacoby, 2015). QnrC is also associated with *P. mirabilis* (Rodríguez-Martínez et al., 2011; Hooper &

Jacoby, 2015). *P. mirabilis* may harbour *aac(6')-Ib-cr,* although it is less frequent than *qnr* genes (Mokracka, Gruszczyńska & Kaznowski, 2012; Ramirez et al., 2013; Zhang et al., 2013). Some studies in companion animals report high *P. mirabilis* enrofloxacin-resistance frequencies (Harada et al., 2014; Criel et al., 2015). Guillard et al. (2016) and de Jong et al. (2018) showed that quinolone-resistance in *P. mirabilis* from companion animals in Europe is mainly associated with QRDR mutations. The same studies also detected *qnrD* and less frequently *qnrS* PMQRs (Guillard et al., 2016; de Jong et al., 2018). The *qnrD* sequences from companion animal *P. mirabilis* were similar to previous published data about isolates from humans (Guillard et al., 2016). Similar distribution of resistance determinants was reported in earlier studies of companion animals (Zhang et al., 2013; Harada et al., 2014; Schultz et al., 2017a). Additionally, *qnrA* was found as part of a SGI1 in *P. mirabilis* from dogs (Schultz et al., 2015). Although, *aac(6')-Ib-cr* is rare in *P. mirabilis*, it has been detected in companion animals (Zhang et al., 2013).

1.5.2.3 Other antimicrobials

P. mirabilis is naturally susceptible to trimethoprim/sulfamethoxazole, however studies from human and companion animal clinical strains tend to report high frequencies of resistance (Szabo & Paterson, 2002; Stock, 2003; Pedersen et al., 2007; Harada et al., 2014; Kroemer et al., 2014; Wang et al., 2014; Criel et al., 2015; Moyaert et al., 2016). Likewise, *P. mirabilis* also usually have high frequencies of chloramphenicol-resistance (Stock, 2003; Harada et al., 2014). There is a lack of studies focussed on determining the frequency of trimethoprim and sulfonamide-resistance mechanisms in *P. mirabilis*. Nevertheless, some mobile resistance determinants described in *P. mirabilis* have *sul and dfr* genes in its composition, such as the SXT/R391-related ICEs, SGI1, PGI1 and class 1 integrons (Grape, Farra, Kronvall & Sundström, 2005; Siebor & Neuwirth, 2014; Schultz et al., 2015; Aogáin et al., 2016, Li et al., 2016). Overall, *sul1*, *sul2* and *sul3* have been detected in *P. mirabilis* of different sources (Grape, Farra, Kronvall & Sundström, 2005; Siebor & Neuwirth, 2014; Schultz et al., 2015; Li et al., 2016; Jiang et al., 2017; Siebor, de Curraize & Neuwirth, 2018).

The frequency of chloramphenicol-resistance mechanism in *P. mirabilis* is also poorly studied. However, there are reports of mobile chloramphenicol-resistance mechanisms in *P. mirabilis* such as *cmlA* and *floR* (Li et al., 2016; Jiang et al., 2017; Siebor et al., 2018).

According to Ramirez and Tolmasky (2010) review, *P. mirabilis* may harbour AAC(3)-Ia, AAC(3)-Id, AAC(3)-Ie and AAC(6′)-Ib AMEs. Posterior studies have shown that *P. mirabilis* may also carry additional AMEs (Miró et al., 2013b; Aogáin et al., 2016; Chen, Lai, Wu & Wu, 2017; Siebor et al., 2018). 16S rRNA methylases have also been detected among *P. mirabilis* (Yu et al., 2010; Wachino et al., 2012). Interestingly, in a study regarding class 1 integrons, Chen et al. (2017) showed that the most frequent gene cassetes in *Proteus mirabilis* isolates from urine samples in China contained resistance mechanisms against aminoglycosides

(*aac(6')-Ib, aacA7, aadAI, aadA2,* and *aadAIa*), trimethoprim (*dfrAI* and *dfrA12*) and chloramphenicol (*catB* and *cmlA*).

1.5.3 Virulence

Despite being considered an opportunistic bacteria, *P. mirabilis* genome is known to encode a wide range of virulence factors (Pearson et al., 2008; Armbruster & Mobley, 2012) (Figure 8). Several of these *P. mirabilis* virulence factors have been shown to play an important role in uropathogenesis (Coker, Poore, Li & Mobley, 2000; Pearson et al., 2008; Armbruster & Mobley, 2012) and to be possible vaccine candidates or therapeutic targets (Schaffer & Pearson, 2015; Armbruster et al., 2018). Regarding companion animals, the frequency or characterisation of virulence factors of *P. mirabilis* has been seldomly addressed (Bijlsma, van Dijk, Kusters & Gaastra, 1995; Gaastra et al., 1996).

1.5.3.1 Fimbriae (Pilli)

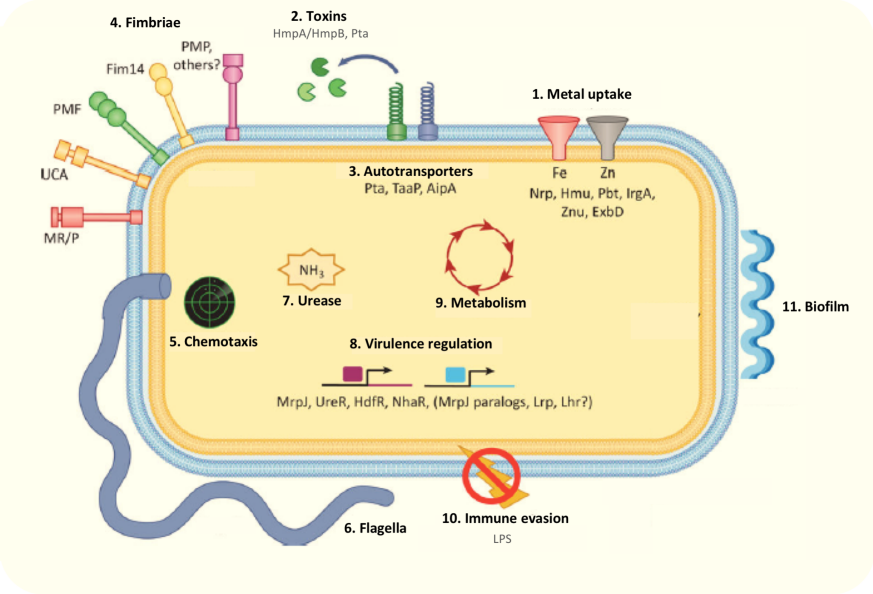
Analysis of the first *P. mirabilis* complete genome sequence revealed a total of 17 potential chaperone–usher fimbrial operons (Pearson et al., 2008). Kuan, Schaffer, Zouzias and Pearson (2014) noted that 16 of these were encoded by most *P. mirabilis,* thus being likely highly conserved. Only five of these 17 fimbriae had been characterised at the time, of which three were known to contribute to uropathogenesis, namely mannose-resistant *Proteus*-like fimbriae (MR/P), urothelial cell adhesion fimbriae (UCA/NAF, also known as non-agglutinating fimbriae) and *Proteus mirabilis* fimbriae (PMF) (Rocha, Pelayo & Elias, 2007; Pearson et al., 2008; Schaffer & Pearson, 2015). These fimbriae possibly act in concert to optimise the urinary tract colonisation (Massad, Bahrani & Mobley, 1994a; Zunino et al., 2007).

The MR/P fimbriae is the best characterised *P. mirabilis* fimbriae (Schaffer & Pearson, 2015; Armbruster et al., 2018). Several studies have demonstrated that MR/P fimbriae undergoes phase variation and that it plays an important role in uropathogenesis (Jansen, Lockatell, Johnson & Mobley, 2004; Bahrani & Mobley,1994; Rocha, Pelayo & Elias, 2007; Schaffer & Pearson, 2015; Armbruster et al., 2018). Notably, MR/P fimbriae were found to be highly expressed by most *P. mirabilis* during an ascending UTI murine model (Jansen et al., 2004; Pearson, Yep, Smith & Mobley, 2011) and are also expressed during pyelonephritis (Mobley & Chippendale, 1990). The MR/P gene cluster is constituted by the *mrp* operon (*mrpABCDEFGHJ*) which has several similarities with *E. coli Fim* and *Pap* fimbriae genes (Li, Johnson & Mobley, 1999; Bahrani & Mobley,1994). MR/P fimbriae promotes initial biofilm production, bind to uroepithelial cells and are essential for colonisation of the bladder and kidneys (Sareneva, Holthöfer & Korhonen, 1990; Jansen et al., 2004; Schaffer & Pearson, 2015; Armbruster et al., 2018).

First described in 1986, the UCA/NAF fimbriae is organised as long and flexible rods and is encoded in an operon containing 5 genes (Wray, Hull, Cook, Barrish & Hull, 1986; Rocha et

al., 2007; Pearson et al., 2008). The major subunit *ucaA* gene was found to be more variable than other fimbriae (Kuan et al., 2014). These fimbriae adheres to uroepithelial cells, Madin-Darby canine kidney cells *in vitro* and play an important role in the colonisation of the urinary tract in the mouse model, including towards the kidney (Wray et al., 1986; Cook, Mody, Valle & Hull, 1995; Lee, Harrison, Latta & Altman, 2000; Pellegrino, Scavone, Umpiérrez, Maskell & Zunino, 2013). It is interesting to notice that UCA/NAF fimbriae is able to bind to GalNAcβ1-4Gal like other fimbriae of uropathogenic *E. coli* (Lee, Harrison, Latta & Altman, 2000).

Figure 8 – *P. mirabilis* virulence factors (adapted from Norsworthy and Pearson, 2017)



Legend: 1. Metal uptake: systems of iron and zinc scavenging; 2. Toxins: proteins such as HpmA/HpmB (haemolysin) and Pta (*Proteus* toxic agglutinin); 3. Autotransporter: such as Taap (trimeric autoagglutination autotransporter of *Proteus*, PMI2575), AipA (adhesion and invasion mediated by the *Proteus* autotransporter, PMI2122) and Pta; 4. Fimbriae: such as MR/P (mannose-resistant *Proteus*-like fimbriae), UCA (urothelial cell adhesion fimbriae), PMF (*Proteus mirabilis* fimbriae), FIM14 (Fimbriae 14) and PMP (*P. mirabilis* P-like); 5. Chemotaxis: proteins that allow the bacteria to follow chemical gradients; 6. Flagella: needed for swarming motility; 7. Urease: key contributor for stone formation during UTI; 8. Virulence regulation: essential for the coordination of all steps of infection; 9. Metabolism: likely needed to support nutritional niches, compete with other bacteria and interact with the host cues; 10. Immune evasion: includes several factors, such as antibody and antimicrobial peptide degradation and LPS (lipopolysaccharide); 11. Biofilm formation: associated with persistence and antimicrobial resistance.

The PMF fimbriae is encoded in an operon composed by five functional genes (*pmfACDEF*) where the *pmfA* encodes the major structural subunit (Massad & Mobley, 1994). The PMF fimbriae has a role in the bladder colonisation *in vivo*, however studies do not agree regarding to its role in kidney colonisation (Massad, Lockatell, Johnson & Mobley, 1994b; Zunino et al., 2003). Despite MR/P and PMF fimbriae having specific and additive roles during *P. mirabilis* UTI (Zunino et al., 2007), Pearson et al. (2011) surprisingly found that *pmfA* is downregulated during UTI.

Kuan et al., 2014 published that most *P. mirabilis* fimbriae belong to 3 groups of the Greek classification. The MR/P fimbriae belongs to the same group as *E. coli* type-1 fimbriae (associated with UTI) while the UCA/NAF and PFM fimbriae belongs to the same group as the uropathogenic *E. coli* P fimbriae (associated with pyelonephritis).

The two-other characterised *P. mirabilis* fimbriae for which no relation with uropathogenesis has been found include the *P. mirabilis* P-like (PMP) fimbriae and ambient-temperature fimbriae (ATF). The PMP fimbriae was first detected in *P. mirabilis* from dogs with UTI and was present in high frequency (Bijlsma et al., 1995; Rocha et al., 2007). Since then it has been found in the genome of strains from humans, but its role in pathogenesis is unclear (Pearson et al., 2008; Baldo & Rocha, 2014). The ATF fimbriae has its optimal expression at 23ºC and seems to have a role in *P. mirabilis* environmental lifestyle (Massad et al., 1994a; Rocha et al., 2007). Zunino, Geymonat, Allen, Legnani-Fajardo and Maskell (2000) demonstrated that the *P. mirabis* ATF fimbriae does not play a crucial role in UTI.

Early studies also describe an additional fimbria, the mannose-resistant *Klebsiella*-like (MR/K) fimbriae that was found to adherence to the Bowman's capsules of the kidney glomeruli, to the tubular basement membranes and to the interstitial tissue (Sareneva et al., 1990; Rocha et al., 2007). This fimbria is poorly studied because its encoding genes have not been identified (Rocha et al., 2007; Kuan et al., 2014).

The MR/P, UCA/NAF and PMF fimbriae seem to be highly frequent among *P. mirabilis* from humans regardless of the isolation site (Old & Adegbola, 1982; Mobley & Chippendale, 1990; Sosa, Schlapp & Zunino, 2006; Kuan et al., 2014). Regarding companion animals, Gaastra et al. (1996) showed that *P. mirabilis* from urine and faecal samples of dogs with recurrent UTI were all positive for MR/P and almost all positive for UCA/NAF fimbriae. Bijlsma et al. (1995) also detected the UCA/NAF fimbriae among *P. mirabilis* from dogs. Furthermore, Garcia, Hamers, Bergmans, van der Zeijst and Gaastra (1988) showed that the uropathogenic *P. mirabilis* adhesion to dog kidney epithelial cells and T24 cells was not species specific when comparing isolates from dogs (provided that *P. mirabilis* was fimbriated) and humans.

1.5.3.2 Urease

One hallmark of *P. mirabilis* UTI pathogenesis is the formation of urinary stones (Baldo & Rocha, 2014). This leads to adverse consequences for the infected patient (e.g. blockage of urinary flow, catheter blockage, etc.) and provides a protective environment for *P. mirabilis* (Armbruster & Mobley, 2012; Baldo & Rocha, 2014; Schaffer & Pearson, 2015; Armbruster et al., 2018). Urinary stones may also be used by other bacteria as a starting point to develop UTI (Armbruster & Mobley, 2012; Schaffer & Pearson, 2015; Armbruster et al., 2018). In dogs, uroliths have been found to sometimes be colonised by varied bacteria genus despite negative urine culture (Gatoria, Saini, Rai & Dwivedi, 2006). Stone formation is driven by increased urine pH which leads to the precipitation of calcium and magnesium ions with the subsequent

formation of magnesium ammonium phosphate (struvite) and calcium phosphate (apatite) stones (Griffith & Musher, 1976). The increased urine pH results from the activity of *P. mirabilis* urease by hydrolysing urea into ammonia and carbon dioxide (Griffith & Musher, 1976). The urease is a multimeric nickel-metalloenzyme encoded by *ureRDABCEFG* gene cluster which expression is induced and upregulated during UTI (Zhao, Thompson, Lockatell, Johnson & Mobley, 1998; Pearson et al., 2008; Pearson et al., 2011). In fact, urease is a crucial virulence factor in *P. mirabilis* colonisation, urolithiasis and severe acute pyelonephritis (Mobley, Chippendale, Swihart & Welch, 1991; Johnson et al., 1993). Interestingly, the resulting increased ammonia content in the urine seems to enhance the colonisation by uropathogenic *E. coli* (Armbruster & Mobley, 2012). Due to the relevant role of urease and pH increase in the pathogenesis of UTI, these factors are actively investigated in the pursue of new therapeutic options (Schaffer & Pearson, 2015).

1.5.3.3 Haemolysin

P. mirabilis produces a calcium-independent haemolysin that is a two-partner secretion system, HpmA and HpmB, where HpmB transports and activates HpmA (Swihart & Welch, 1990a; Uphoff & Welch, 1990; Coker et al., 2000). HpmA has cytotoxic activity onto several types of cells *in vitro*, including T24 human bladder epithelial cells and human renal proximal tubular epithelial cells (Peerbooms, Verweij & MacLaren, 1983; Swihart & Welch, 1990a; Mobley et al., 1991). HmpA did not seem to affect the *P. mirablis* kidney colonisation or histologic damage *in vivo* (Swihart & Welch, 1990a; Mobley et al., 1991), however, one HmpA-mutant *P. mirabilis* had a six-fold lower 50% lethal dose in mice injected intravenously (Swihart & Welch, 1990a). Interestingly, *hmpA* was not found to be upregulated during UTI (Pearson et al., 2011). Some authors hypothesise that despite not being involved in colonisation, haemolysin may contribute to the spread of infection into the kidneys and lead to acute pyelonephritis (Coker et al., 2000).

Most *P. mirabilis* are haemolytic and encode for HpmA/HpmB, including those causing human UTI (Mobley & Chippendale, 1990; Swihart & Welch, 1990b; Sosa et al., 2006; Cestari et al., 2013). *P. mirabilis* from companion animals are also frequently haemolytic (Gaastra et al., 1996). Although no study has tested for the presence of HpmA/HpmB genes in *P. mirabilis* strains from companion animals, based on current knowledge, one could expect it to be equally frequent.

1.5.3.4 Other toxins

Early studies and recent sequencing data shows that *P. mirabilis* genome encodes several known and putative toxins (Armbruster & Mobley, 2012).

Proteus toxic agglutinin (Pta) is a bifunctional outer-membrane autotransporter that mediates cell–cell aggregation and acts as a cytotoxic protease capable of lysing kidney and bladder

cells (Alamuri & Mobley, 2008; Alamuri, Eaton, Himpsl, Smith & Mobley, 2009). Pta is further noteworthy in the scope of UTI because loss of this gene significantly reduces *P. mirabilis* colonisation of the bladder, kidneys and spleen (Alamuri & Mobley, 2008; Alamuri et al., 2009). Pta and HmpA seem to have addictive cytotoxicity (Alamuri et al., 2009) and Pta has higher expression in alkaline pH (Alamuri & Mobley, 2008).

1.5.3.5 Swarming motility

The swarming ability of *P. mirabilis* is a complex process that has been extensively studied and is recognised as an important virulence factor (Coker et al., 2000; Armbruster & Mobley, 2012; Schaffer & Pearson, 2015). Swarming involves *P. mirabilis* differentiation into swarmer cells that are long, polyploid, hyperflagelated and able to move as a population on a solid surface (Schaffer & Pearson, 2015). The classic bull's-eye pattern results from cyclic differentiation between swarm cells, which are capable of migration, and a shorter morphotype (swimmer cell) (Manos & Belas, 2006; Armbruster & Mobley, 2012; Schaffer & Pearson, 2015). There are conflicting results in the literature regarding the role of swarming in uropathogenesis (Schaffer & Pearson, 2015; Armbruster et al., 2018). This phenomenon is considered important in the uropathogenic nature of *P. mirabilis* since it assists in urinary catheter colonisation and has the potential to influence polymicrobial UTIs (Armbruster & Mobley, 2012; Schaffer & Pearson, 2015). Interestingly, fimbriae and flagella regulation seem to be reciprocal since *P. mirabilis* highly express either one or the other in swimmer and swarmer cells, respectively (Armbruster & Mobley, 2012). Non-flagelated *P. mirabilis* mutants were attenuated in an experimental model of ascending UTI (Allison, Emödy, Coleman & Hughes, 1994; Mobley et al., 1996). Based on expression studies, swarmer cell may be important at later stages of UTI infection (Armbruster & Mobley, 2012; Schaffer & Pearson, 2015).

1.5.3.6 Other virulence factors

P. mirabilis has additional virulence factors that may contribute to UTI, such as in assisting immune evasion (Baldo & Rocha, 2014; Armbruster et al., 2018). For instance, the ZapA metalloproteinase cleaves serum/secretory immuno-globulins and complement components and its absence reduces the recovery of *P. mirabilis* from urine, bladder and kidneys (Armbruster & Mobley, 2012; Schaffer & Pearson, 2015; Armbruster et al., 2018). Another example is the AipA (adhesion and invasion mediated by the *Proteus* autotransporter, PMI2122) and the TaaP (trimeric autoagglutination autotransporter of *Proteus*, PMI2575) trimeric autotransporters which absence decreases the urinary tract colonisation by *P. mirabilis* (Schaffer & Pearson, 2015; Armbruster et al., 2018). The formation of crystalline biofilms is also a major *P. mirabilis* virulence factor, especially in catheterised patients (Armbruster et al., 2018). *P. mirabilis* iron acquisition systems have been less studied than in other bacteria, but also seem to be important during UTI (Armbruster & Mobley, 2012). Notably, *P. mirabilis* may

harbour a mobile pathogenicity island (ICE*pm1*), encoding several virulence genes, that was found to be more frequent among isolates from UTI (Flannery, Mody & Mobley, 2009; Flannery, Antczak & Mobley, 2011; Armbruster et al., 2018). On the contrary, other studies have found that several virulence factors involved in UTI (e.g. fimbriae, haemolysin, swarming) were equally common among *P. mirabilis* from different sources (Peerbooms, Verweij, Oe & MacLaren, 1986; Sosa et al., 2006).

1.6 Animal-human transfer and colonisation by *K. pneumoniae* and *P. mirabilis*

Over the last 50 years the number of companion animals has significantly increased (Guardabassi et al., 2004; Pomba et al., 2017). The closer contact between owners and companion animals created opportunities for pathogen interchange through direct and indirect contact (Guardabassi et al., 2004; Damborg et al., 2016). With the increasing resistance trends and detection of MDR bacteria in companion animals, a concern regarding the role of companion animals in the dissemination of resistant bacteria has been raised (Guardabassi et al., 2004; Damborg et al., 2016; Pomba et al., 2017). These concerns are reinforced by studies that have shown that companion animals may share bacteria with household members and studies reporting colonised and/or infected companion animals with bacteria belonging to important clonal lineages to humans (Guardabassi et al., 2004; Damborg et al., 2016; Pomba et al., 2017). As examples, companion animals have been found to be colonised by CC17 *E. faecium*[11], MRSA and ST131 *E. coli* (Guardabassi et al., 2004; Damborg et al., 2009; Weese, 2010; Johnson et al., 2016; Pomba et al., 2017). As mentioned previously, several studies on resistant *K. pneumoniae* detected high-risk clonal lineages to humans infecting companion animals (some with UTI) (Table 6). Furthermore, dogs may share ESBL/AmpC and carbapenemase-producing *E. coli* with their household members (Ljungquist et al., 2016; Grönthal et al., 2018). Just recently, Grönthal et al. (2018) demonstrated that two dogs with otitis shared NDM-5-producing *E. coli* strains with one human living in the same household.

Since uropathogenic bacteria, including *K. pneumoniae* and *P. mirabilis*, are thought to make part of the normal gut flora (Podschun & Ullmann, 1998; Drzewiecka, 2016; Johnson et al., 2016; Martin et al., 2016), colonisation of companion animals may also represent an important hazard for the transmission and persistence of uropathogens. Interestingly, pet ownership (dogs, cats and other companion animals) was suggested to be a risk factor for human gut colonisation by ESBL-producing *E. coli* (Meyer, Gastmeier, Kola & Schwab, 2012). Regarding UTI, companion animals from the same household may be colonised and share uropathogenic *E. coli* of the O25b:H4-B2-ST131 clonal lineage (Johnson, Miller, Johnston, Clabots & DebRoy, 2009; Johnson et al., 2016). The transfer of bacteria from humans to companion animals is also a concern in an animal health perspective. In fact, humans with UTI may share the index uropathogenic *E. coli* with household members including the family dogs and cats (Murray,

[11] *E. faecium* CC 17 is a hospital-adapted clonal complex associated with human infection (Damborg et al., 2009).

Kuskowski & Johnson, 2004; Johnson & Clabots, 2006; Damborg et al., unpublished data[12]). Little is known about *K. pneumoniae* and *P. mirabilis* when compared with *E. coli*. However, understanding the colonisation of healthy and infected companion animals is crucial to clarify the role of dogs and cats as reservoir of *K. pneumoniae* and *P. mirabilis*.

1.6.1 *K. pneumoniae* gut colonisation

K. pneumoniae spreads easily through person-to-person contact (healthcare workers, patients) and contaminated surfaces and instrumentation (Martin & Bachman, 2018). In the colonised human, *K. pneumoniae* can be found mainly in the nasopharynx and the gastrointestinal tract (Podschun & Ullmann, 1998). The gastrointestinal colonisation is believed to be the major *K. pneumoniae* reservoir and is associated with increased risk for subsequent infections in hospitalised patients, including UTIs (Podschun & Ullmann, 1998; Martin et al., 2016; Gorrie et al., 2017). Notably, there is high concordance between the colonising and subsequent infection strains (Martin et al., 2016; Gorrie et al., 2017). This is particularly relevant regarding ESBL/carbapenemase-producing *K. pneumoniae* and regarding hypervirulent strains due to limited therapeutic options and/or high morbidity (Gorrie et al., 2017; Martin & Bachman, 2018). Colonisation rates vary according to the study and studied population (Podschun & Ullmann, 1998; Martin & Bachman, 2018). Nasopharingeal colonisation by *K. pneumoniae* in humans varies from 3 to 15% in community settings and 19% in hospitals (Podschun & Ullmann, 1998; Martin & Bachman, 2018). Reported gastrointestinal colonisation in humans can reach 35% in the community and 20-77% in hospitalised patients (Podschun & Ullmann, 1998; Martin & Bachman, 2018). The higher colonisation rates in hospital settings are associated with higher lengths of stay, working hospital personal and antimicrobial use, among others (Podschun & Ullmann, 1998; Martin & Bachman, 2018). Most *K. pneumoniae* colonisation studies are focussed on hospital settings; however, few reports have also suggested the possible transmission of *K. pneumoniae* between humans within the same household (Gottesman, Agmon, Shwartz & Dan, 2008; Löhr et al., 2013). Little is known about the colonisation rates in dogs and cats and studies regarding animal-human transmission are lacking. Although most studies were focussed on ESBL/carbapenemase-producing bacteria, *K. pneumoniae* has been detected in faecal samples from healthy and diseased dogs (Olson, Hedhammar, Faris, Krovacek & Wadström, 1985; Balish, Cleven, Brown & Yale, 1977; Ewers et al., 2014b; González-Torralba et al., 2016; Sabbioni et al., 2016; Sharif, Sreedevi, Chaitanya & Sreenivasulu, 2017; Silva et al., 2018). Balish et al. (1977) detected a 57% *K. pneumoniae* gut colonisation in enclosed healthy beagles while Shariff et al. (2017) reported 26% of colonised healthy dogs in India. The persistent colonisation of a dog by a CTX-M-15-producing *K. pneumoniae* ST231 after recovery of a respiratory infection and pyometra was recently described by Silva et al. (2018).

[12] Available as a preprint at bioRxiv at https://www.biorxiv.org/content/early/2018/04/17/302885.

1.6.2 *P. mirabilis* gut colonisation

The gut is also suggested as a reservoir of *Proteus* spp. bacteria (Drzewiecka, 2016). Interestingly, Peerbooms et al. (1986), showed that faecal *P. mirabilis* have similar virulence traits as strains causing UTI in humans. Faecal colonisation frequencies of bacteria in humans vary according to the intestinal section considered and *Proteus* spp. ranges from 12% to 45% (Zilberstein et al., 2007). Lower frequencies of *P. mirabilis* colonisation (~2%) have been reported (Müller, 1986; Porres-Osante, Sáenz, Somalo & Torres, 2015). Several studies have pointed to the gut colonisation as a source of uropathogenic *P. mirabilis* strains in human patients with UTI (Drzewiecka, 2016). In fact, catheterised human patients are usually colonised in the gut by the same uropathogenic *P. mirabilis* stain causing UTI (Mathur, Sabbuba, Suller, Stickler & Feneley, 2005). Gaastra et al. (1996) did not detect *P. mirabilis* in the faeces of healthy dogs, however, dogs with UTI were shown to have a high burden of the uropathogenic *P. mirabilis* in urine and faeces. Hariharan et al. (2010) found 6% *P. mirabilis* positive faecal samples in feral cat from Grenada and Balish et al. (1977) detected a 43% *P. mirabilis* gut colonisation in enclosed healthy beagles. Studies addressing the sharing and transmission of *P. mirabilis* between companion animals and humans are lacking.

Chapter 2

Study goals

The major goal of this thesis was to contribute to the understanding of the antimicrobial resistance epidemiology of UTI pathogens in companion animals and its public health relevance, with a special focus to *K. pneumoniae* and *P. mirabilis* species. The experimental work was divided in three main parts:

1 In the first part, the aim of the work was to obtain up-to-date epidemiological information on antimicrobial resistance of bacteria isolated from companion animals with UTI. This goal was pursued by determining the prevalence of uropathogens and antimicrobial resistance across several European countries. Furthermore, the secular temporal trends of antimicrobial resistance and the public health relevance of bacteria harbouring critical antimicrobial resistance genes causing UTI in companion animals from Portugal (Lisbon) was studied.

2 The second part of this work aimed to characterise the molecular epidemiology of clinical *K. pneumoniae* and *P. mirabilis* strains from companion animals and humans with UTI. By studying the population structure, antimicrobial resistance and virulence genotype of strains of both origins, this part of the study aimed to get insights on the role of infected companion animals as reservoirs and in the dissemination of clinical *K. pneumoniae* and *P. mirabilis*.

3 The third part of this thesis aimed to characterise the gut colonisation by *K. pneumoniae* and *P. mirabilis* in healthy companion animals and humans. Through the study of households composed of companion animals and humans living in close contact, this part of the study aimed at providing new data about *K. pneumoniae* and *P. mirabilis* animal-human sharing and assess the role of healthy companion animals as reservoirs to humans and vice versa.

Chapter 3

Epidemiology of uropathogenic bacteria in companion animals

3.1 European multicenter study on antimicrobial resistance in bacteria isolated from companion animal urinary tract infections.

Full paper published at the *BMC Veterinary Research*

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*The author jointly conceived the design of the study and contacted the collaborating laboratories. The author managed all datasets and jointly conducted statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on co-authors and reviewers revisions.

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European multicenter study on antimicrobial resistance in bacteria isolated from companion animal urinary tract infections.

Cátia Marques[1], Luís Telo Gama[1], Adriana Belas[1], Karin Bergström[2], Stéphanie Beurlet[3], Alexandra Briend-Marchal[3], Els M. Broens[4], Marta Costa[5], Delphine Criel[6], Peter Damborg[7], Marloes A. M. van Dijk[4], Astrid M. van Dongen[8], Roswitha Dorsch[9], Carmen Martin Espada[10], Bernhard Gerber[11], Maria Kritsepi-Konstantinou[12], Igor Loncaric[13], Domenico Mion[14], Dusan Misic[15], Rebeca Movilla[16], Gudrun Overesch[17], Vincent Perreten[17], Xavier Roura[16], Joachim Steenbergen[6], Dorina Timofte[18,19,20], Georg Wolf[21], Renato Giulio Zanoni[14], Sarah Schmitt[22], Luca Guardabassi[7,23], Constança Pomba[1]

[1]Faculdade de Medicina Veterinária, Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Lisboa, Portugal.
[2]The National Veterinary Institute (SVA), Uppsala, Sweden.
[3]Laboratoire Vebiotel, Arcueil, France.
[4]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands.
[5]Department of Clinical Veterinary Sciences, Faculty of Medical and Veterinary Sciences, University of Bristol, Langford, UK.
[6]AML-Medvet laboratory, Antwerp, Belgium.
[7]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark.
[8]Department of Clinical Sciences of Companion Animals, University of Utrecht, Utrecht, The Netherlands.
[9]Clinic of Small Animal Medicine, LMU Munich, Munich, Germany.
[10]Hospital Clínico Veterinario Complutense, Universidad Complutense, Servicio de Microbiologia y Parasitologia, Madrid, Spain.
[11]Vetsuisse Faculty, University of Zurich, Clinic for Small Animal Internal Medicine, Zurich, Switzerland.
[12]Department of Clinical Studies, Companion Animal Clinic, Faculty of Health Sciences, School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece.
[13]Institute of Microbiology, University of Veterinary Medicine, Vienna, Austria.
[14]Department of Veterinary Medical Science, University of Bologna, Bologna, Italy.
[15]Department of microbiology, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia.
[16]Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain.
[17]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland.
[18]School of Veterinary Science, University of Liverpool, Cheshire, UK.
[19]Faculty of Veterinary Medicine, University of Agronomical Sciences Veterinary Medicine, Lasi, Romania.
[20]Institute of Infection and Global Health, University of Liverpool, Liverpool, UK.
[21]Institute for Infectious Diseases and Zoonoses, LMU Munich, Munich, Germany.
[22]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.
[23]Department of Biomedical Sciences, Ross University School of Veterinary Medicine, Basseterre, St Kitts and Nevis.

Abstract

Background: There is a growing concern regarding the increase of antimicrobial resistant bacteria in companion animals. Yet, there are no studies comparing the resistance levels of these organisms in European countries. The aim of this study was to investigate geographical and temporal trends of antimicrobial resistant bacteria causing urinary tract infection (UTI) in companion animals in Europe. The antimicrobial susceptibility of 22 256 bacteria isolated from dogs and cats with UTI was determined. Samples were collected between 2008 and 2013 from 16 laboratories of 14 European countries. The prevalence of antimicrobial resistance of the most common bacteria was determined for each country individually in the years 2012–2013 and temporal trends of bacteria resistance were established by logistic regression.

Results: The aetiology of uropathogenic bacteria differed between dogs and cats. For all bacterial species, Southern countries generally presented higher levels of antimicrobial resistance compared to Northern countries. Multidrug-resistant *Escherichia coli* were found to be more prevalent in Southern countries. During the study period, the level of fluoroquinolone-resistant *E. coli* isolated in Belgium, Denmark, France and the Netherlands decreased significantly. A temporal increase in resistance to amoxicillin/clavulanate and gentamicin was observed among *E. coli* isolates from the Netherlands and Switzerland, respectively. Other country-specific temporal increases were observed for fluoroquinolone-resistant *Proteus* spp. isolated from companion animals from Belgium.

Conclusions: This work brings new insights into the current status of antimicrobial resistance in bacteria isolated from companion animals with UTI in Europe and reinforces the need for strategies aiming to reduce resistance.

Keywords: Antimicrobial resistance, Temporal trends, MRSA, MRSP, Dog, Cat

Introduction

Bacterial urinary tract infections (UTI) are frequently diagnosed in dogs and are considered rare in cats (Dowling, 1996; Thompson et al., 2011). Lately, increased frequencies of UTI in cats have been reported in some European countries (Gerber et al., 2005; Eggertsdóttir et al., 2007; Sævik et al., 2011) in particularly when concurrent diseases are present (Mayer-Roenne et al., 2007).

Escherichia coli is the most frequent isolated bacteria causing UTI in dogs and cats. Several studies show frequencies greater than 30% (Litster et al., 2007b; Hall et al., 2013; Dorsch et al., 2015). Other commonly isolated bacteria genera include *Staphylococcus* spp., *Enterococcus* spp., *Proteus* spp. and *Klebsiella* spp. (Féria et al., 2000; Litster et al., 2007b; Hall et al., 2013; Dorsch et al., 2015).

Previous studies in the United Kingdom and in Missouri-Columbia (USA) analysing the temporal trends of antimicrobial resistance in small collections of bacterial isolates from companion animal infections point to a significant increase in antimicrobial resistance (Normand et al., 2000a; Cohn et al., 2013). Furthermore, the emergence of multidrug-resistant bacteria (isolates resistant to three or more antimicrobial categories) in companion animals is an increasing concern (Pellerin et al., 1998; Normand et al., 2000a; Pomba et al., 2014a; Pomba et al., 2017). This creates new therapeutic challenges in veterinary medicine and is also a public health issue, since these pathogens may be zoonotic (Johnson & Clabots, 2006) and companion animals may play a role in the spread of resistant bacteria due to their close contact to humans (Guardabassi et al., 2004; Pomba et al., 2017).

Antimicrobial resistance may vary according to the geographic location (European Centre for Disease Prevention and Control [ECDC], 2014; Dorsch et al., 2015). Data on antimicrobial resistance in bacteria isolated from companion animals with UTI in Europe are not easily comparable due to differences in study design, such as variations in host species, inclusion criteria and/or time period. Thus, it is difficult to get a European overview of antimicrobial resistance as seen in human medicine surveillance programmes such as the European Antimicrobial Resistance Surveillance Network (ECDC, 2014).

Antimicrobial therapy in UTI should ideally rely on susceptibility testing of the isolated bacteria (Weese et al., 2011). Yet, antimicrobials are frequently administered empirically based on the presence of compatible clinical signs, urine cytological findings and in the absence of urine culture and are required to alleviate UTI symptoms while waiting for antimicrobial susceptibility testing results (Weese et al., 2011). Besides the pharmacokinetic-pharmacodynamic properties, the empiric antimicrobial selection should consider the most likely causative agent as well as its regional susceptibility patterns (Hall et al., 2013). Moreover, according to the World Organization for Animal Health (World Organization for Animal Health [OIE], 2018), veterinarians should adopt strategies aimed at the reduction of antimicrobial resistance. Therefore, current information on the aetiology and antimicrobial resistance focussed on UTI is of crucial importance.

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. A Urinary Tract Infection Resistance – Veterinary Network (UTIR-VNet) was constituted with this purpose in mind. Partial results were presented at the annual Society meeting included in the 25th congress of the European College of Veterinary Internal Medicine, 4–6 September 2014, Mainz, Germany. The aim of this study was to determine the frequency of uropathogens in dogs and cats with urinary tract infection in Europe and to characterise the frequency and temporal trends of antimicrobial resistance over a period of six years. We hereby present a complete report and discussion of this study.

Methods

Participating countries

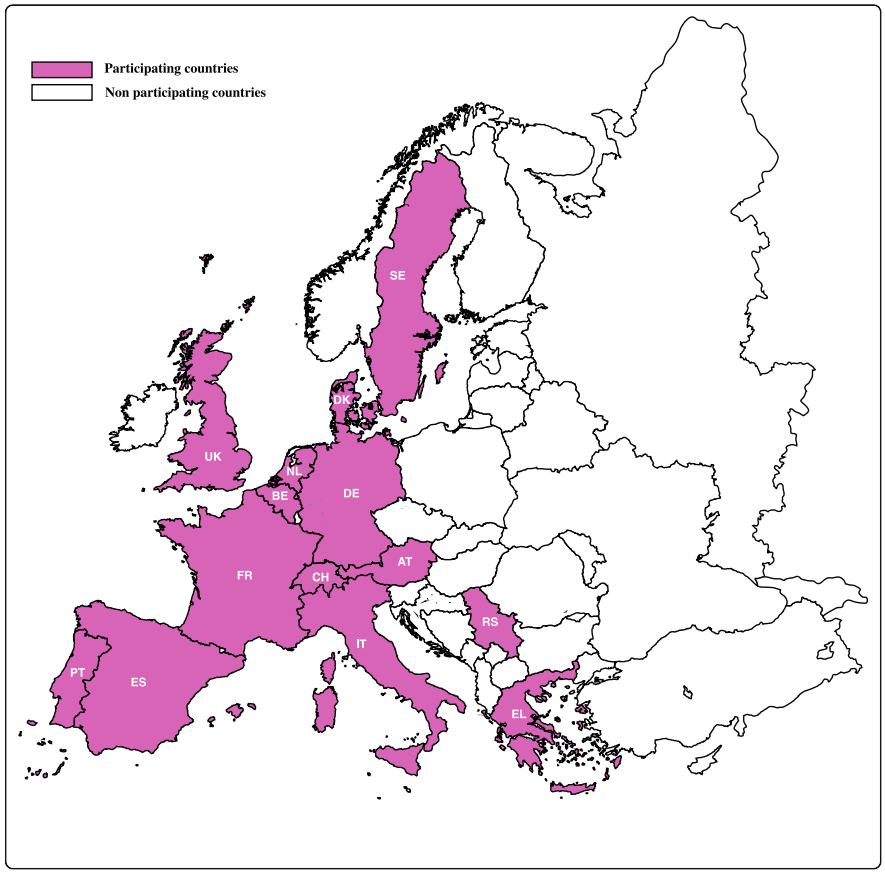
Between January and September 2014, 16 veterinary microbiology laboratories from 14 European countries (Austria, Belgium, Denmark, France, Germany, Greece, Italy, the Netherlands, Portugal, Serbia, Spain, Sweden, Switzerland, United Kingdom), were invited to participate in this study (Figure 1). Laboratories were requested to send available retrospective data on animal species, age and gender, bacterial identification and antimicrobial susceptibility testing conducted in bacteria obtained from dogs and cats with UTI between 2008 and 2013.

Samples were obtained with owners consent as part of the routine care of canine and feline UTI.

Bacterial Isolates

The bacteria identification varied between laboratories. Most laboratories used standard phenotypic tests, including API, while others used techniques such as PCR and MALDI-TOF. This discrepancy was particularly evident for staphylococci, which were classified to either the species or genus level depending on the laboratory.

Figure 1 - Participating countries in the urinary tract infection antimicrobial resistance veterinary network – UTIR-VNet



Legend: 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

Susceptibility testing

The following antimicrobials were included: amoxicillin/clavulanate (AMC), ampicillin (AMP), cefotaxime (CTX), cefovecin (CVN), cefoxitin (FOX), ceftazidime (CAZ), cefpodoxime (CPD), ceftiofur (EFT), ciprofloxacin (CIP), enrofloxacin (ENR), gentamicin (CN), marbofloxacin (MAR), oxacillin (OX), penicillin (P) and trimethoprim/sulfamethoxazole (SXT).

The retrospective nature of the study forced us to include two in vitro antimicrobial susceptibility testing methods. Laboratories from Austria, France, Germany, Greece, Italy, the Netherlands,

Portugal, Serbia, Spain, United Kingdom, used standard disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute [CLSI], 2013b), whereas Sweden (VetMIC, SVA, Uppsala, Sweeden), Denmark and United Kingdom (COMPAN1F Sensititre panels, Thermo Fisher), Switzerland and Belgium (VITEK 2, BioMérieux) used broth microdilution method.

Human CLSI breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2015) were used for interpretation of minimal inhibitory concentration and disk diffusion results for CAZ (30 µg), CTX (30 µg), and CIP (5 µg), whereas veterinary CLSI breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2013b) were used for AMC (30 µg), AMP (30 µg), CN (10 µg), CPD (10 µg), ENR (5 µg), FOX (30 µg), MAR (5 µg), OX (1 µg), P (10U), and SXT (25 µg). Clinical breakpoints from the Societé Française de Microbiology (Societe Française de Microbiology [SFM], 2012) were used for EFT (30 µg). Results for CVN (30 µg) were interpreted according to the manufacturer guidelines. As seen in the human European Antimicrobial Resistance Surveillance Network (EARS-Net) report (ECDC, 2014), when data on minimal inhibitory concentrations or inhibition zone diameter were not available, the laboratories' own interpretations (susceptible, intermediate and resistant) were accepted. This was the case for Spain, Serbia, and Germany that used contemporary CLSI guidelines, for the United Kingdom that used the breakpoints from the British Society of Antimicrobial Chemotherapy (Howe, Andrews & BSAC Working Party on Susceptibility Testing, 2012) and for the Netherlands and Switzerland (2008–2010) that used breakpoints recommended by the Dutch Committee on Guidelines for Susceptibility testing (Commissie Richtlijnen Gevoeligheidsbepalingen, 2000).

Data analysis and statistical methods

Statistical analysis was performed using the SAS statistical software package for Windows, version 9.3, (SAS Institute Inc, Cary, North Carolina, USA).

The Fisher exact test was used to compare pathogen frequencies by species or gender of the host, by simple/multiple infection and by country. An alpha value of 0.05 was used.

Isolates were considered fully-resistant when found to be resistant according to the clinical breakpoint applied. An isolate was considered susceptible when found to be susceptible or intermediate according to the clinical breakpoint applied. The antimicrobials included in this study are known to be highly concentrated in the urine so their report as susceptible may be appropriated for isolates categorised as intermediate (Weese et al., 2011).

Regarding third-generation cephalosporins (3GC), laboratories tested different 3GC-resistance surrogates. Therefore, to evaluate the antimicrobial resistance to 3GC, an isolate was considered as 3GC-resistant when it was resistant to at least one of the five 3GC tested (CTX, CAZ, CVN, EFT or CPD). The same rational was applied to evaluate resistance to fluoroquinolones (FLU), namely using ENR, CIP or MAR as a marker of resistance. Methicillin-

resistance in staphylococci was determined according to CLSI guidelines (CLSI, 2013a) using cefoxitin or oxacillin to evaluate resistance depending on the bacterial species considered. Yet, Germany and Spain did not send data on methicillin-resistance. France did not test staphylococci against oxacillin and thus did not report methicillin-resistance regarding *Staphylococcus pseudintermedius* (MRSP). The Netherlands did not have data on staphylococci susceptibility to OX or FOX but instead reported data on the detection of the *mecA* gene by PCR. The frequency of methicillin-resistance did not include staphylococci only identified to the genus level.

Enterobacteriaceae were considered multidrug-resistant (MDR) when fully-resistant to three or more categories of antimicrobials, namely AMC, 3GC, SXT, CN and/or FLU. Unlike the MDR definition proposed by other authors (Magiorakos et al., 2012), intermediate isolates from this study were considered as susceptible. This difference was applied because we are considering drugs that can be highly concentrated in urine. Furthermore, this approach will reduce any overestimation of MDR frequency due to the use of different breakpoint guidelines. Full-susceptibility (FullS) was defined as an isolate being susceptible for all the above-mentioned categories of antimicrobials. Since Belgium had no data available on 3GC and the Netherlands had little data on CN, MDR and FullS percentages do not include resistance to 3GC for Belgium and resistance to CN for the Netherlands.

As a rule, statistical analysis was only done when at least ten isolates for a specific organism-antimicrobial agent combination were reported for a given country. All frequencies are presented with a confidence interval of 95% (95% CI).

Maps of European resistance distribution were drawn considering the percentage of fully-resistant isolates to the considered antimicrobial agent, over the years 2012–2013. A scale of colours was applied composed of six resistance intervals after the example of EARS-Net surveillance programme reports (ECDC, 2014).

Statistical analysis of temporal trends of antimicrobial resistance for a specific organism-antimicrobial agent combination were determined within each country. Temporal trends were only determined for countries reporting data on at least three consecutive years and ten isolates per year. A SAS LOGISTIC regression, with the year as a continuous variable and an alpha value of 0.05 was conducted. Temporal trends of resistance were mainly determined for *E. coli* since this was the most represented bacterial species. Yet, temporal trends of AMC, FLU and SXT in *Proteus* spp. were also determined for Belgium, France, the Netherlands and Sweden.

Results

Overall, data on 22256 uropathogenic bacteria were obtained from 15097 dog and 5963 cat positive urine cultures. Table 1 summarises the numbers of bacterial isolates obtained by year and country.

Considering the records containing information about the age, dogs (n = 4425) and cats (n = 1514) had similar mean ages, namely 8.77 years (SD ± 4.04, 9.00 median, 6.00 IQR, range 0.1–20) and 8.82 years (SD ± 5.03, 8.50 median, 8.1 IQR, range 0.2–22) respectively. Gender was only specified in 3885 records where 61.41% (95%CI 59.69–63.12%, n = 1900/3094) of dogs and 48.29% (95% CI 44.81–51.78%, n = 382/791) of cats were females.

Among all urine cultures, 94.64% (95% CI 94.33–94.94%, n = 19932/21060) resulted in the growth of bacterial pure cultures, with no significant difference between cats and dogs (p = 0.1856). Both in dogs and cats, *E. coli* was the most frequently identified bacteria and accounted for 59.45% (95% CI 58.80–60.09%, n = 13231/22256) of all isolates. The frequency of the remaining bacterial species differed significantly between dogs and cats (Table 2). *Enterococcus* spp. and *Staphylococcus* spp. frequencies were higher in cats, whereas *Proteus* spp. and *Klebsiella* spp. were more prevalent in dogs.

Table 1 - Total number of isolated bacteria by year and country

Country[a]	Year						
	2008	2009	2010	2011	2012	2013	Total
AT	-	-	-	-	144	185	329
BE	-	-	547	578	623	739	2487
DK	29	30	53	116	153	205	587
FR	-	-	620	733	780	995	3128
DE	-	64	93	140	161	146	604
EL	24	29	13	11	32	43	152
IT	-	36	29	36	77	65	243
NL	480	867	958	1132	1195	1307	5939
PT	77	54	57	34	32	45	299
RS	17	19	10	2	3	3	54
ES	14	23	27	40	47	79	230
SE	730	924	1071	1202	1355	1647	6929
CH	109	120	112	125	114	174	754
UK	31	44	81	117	126	122	521
Total	1511	2210	3671	4267	4842	5755	22256

Legend: [a]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.

Considering the years 2012–2013, the major differences in *E. coli* (Table 3, Figure 2) and *Proteus* spp. (Table 4, Figure 3) antimicrobial resistance frequencies were seen between Northern (Denmark and Sweden) and Southern (Italy, Greece, Portugal and Spain) countries. The lowest frequencies of AMC resistance in *E. coli* were detected in isolates from Denmark (2.88%) and Belgium (4.29%). *E. coli* from Portugal (48.15%) had a significantly higher AMC

Table 2 - Uropathogenic bacteria aetiology, single versus mixed infections and cat versus dog as host species

Organism	Overall		Single organism		Mixed infections			Dogs		Cats		
	n	**% (95% CI)[a]**	***n***	**% (95% CI)[a]**	***n***	**% (95% CI)[a]**	***P***	***n***	**% (95% CI)[a]**	***n***	**% (95% CI)[a]**	***P***
Enterobacter spp.	308	1.38 (1.23–1.54)	244	1.22 (1.07–1.38)	64	2.75 (2.09–3.42)	**<0.0001**	194	1.21 (1.04–1.38)	114	1.81 (1.48–2.14)	**0.0008**
Enterococcus spp.	1506	6.77 (6.44–7.10)	1129	5.66 (5.34–5.99)	377	16.22 (14.72–17.72)	**<0.0001**	745	4.66 (4.34–4.99)	761	12.11 (11.31–12.92)	**<0.0001**
Escherichia coli	13231	59.45 (58.80–60.09)	12417	62.30 (61.62–62.97)	814	35.03 (33.09-36-97)	**<0.0001**	9506	59.51 (58.75–60.27)	3725	59.30 (58.08–60.51)	0.7832
Klebsiella spp.	478	2.15 (1.96–2.34)	400	2.01 (1.81–2.20)	78	3.36 (2.62–4.09)	**<0.0001**	385	2.41 (2.17–2.65)	93	1.48 (1.18–1.78)	**<0.0001**
Proteus spp.	1992	8.95 (8.58–9.33)	1770	8.88 (8.49–9.28)	222	9.55 (8.36–10.75)	0.2824	1869	11.70 (1.20–1.22)	123	1.96 (1.62–2.30)	**<0.0001**
Pseudomonas spp.	389	1.75 (1.58–1.92)	315	1.58 (1.41–1.75)	74	3.18 (2.47–3.90)	**<0.0001**	293	1.83 (1.63–2.04)	96	1.53 (1.22–1.83)	0.1249
Staphylococcus spp.	2893	13.00 (12.56–13.44)	2519	12.64 (12.18–13.10)	374	16.09 (14.60–17.59)	**<0.0001**	1836	11.49 (11.00–11.99)	1057	16.83 (15.90–17.75)	**<0.0001**
Streptococcus spp.	802	3.60 (3.36–3.85)	586	2.94 (2.71–3.17)	216	9.29 (8.11–10.47)	**<0.0001**	675	4.23 (3.91–4.54)	127	2.02 (1.67–2.37)	**<0.0001**
Other	657	2.95 (2.73–3.17)	552	2.77 (2.54–3.00)	105	4.52 (3.67–5.36)	-	471	2.95 (2.69–3.21)	186	2.96 (2.54–3.38)	-

Legend: [a]95 % CI, 95 % Confidence interval;
n – Total number of isolates;
P - *P* value obtained by Fisher exact test when comparing single versus mixed infections and cat versus dog as host. Statistically significant values are highlighted in bold.

Table 3 - Percentage of resistance in *E. coli* by antimicrobial and country in 2012–2013

Country[a]	AMC		3GC		FLU		CN		SXT		Combined resistance		
	n	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% MDR (95% CI)[b] [Stat. Dif.][c]**	**% FullS (95% CI)[b] [Stat. Dif.][c]**
AT	142	14.08 (8.36–19.81) [a,b]	142	5.63 (1.84–9.43) [a, b]	142	11.97 (6.63–17.31) [a]	142	5.63 (1.84–9.43) [a, b]	142	14.08 (8.36–19.81) [a, b]	142	8.45 (3.88–13.03) [a]	78.87 (72.16–85.59) [a]
BE	840	4.29 (2.92–5.66) [c]	0	- -	769	6.63 (4.87–8.39) [b]	840	1.67 (0.80–2.53) [c]	839	10.37 (8.31–12.43) [a]	769[d]	1.43[d] (0.59–2.27) -	85.05[d] (82.52–87.57) -
DK	206	2.88 (0.61–5.16) [c]	208	4.33 (1.41–7.09) [a, c]	208	2.88 (0.61–5.16) [c]	208	1.92 (0.06–3.79) [a, c]	208	8.17 (4.45–11.90) [a, c]	208	2.88 (0.61–5.16) [b]	88.94 (84.68–93.20) [b, c]
FR	954	12.79 (10.67–15.91) [a, d]	933	10.83 (8.83–12.82) [b]	948	12.76 (10.64–14.89) [a]	951	3.36 (2.22–4.51) [a, d]	959	16.27 (13.93–18.60) [b, d]	909	11.00 (8.97–13.04) [a, c]	77.23 (74.50–79.95) [a]
DE	153	11.76 (6.66–16.87) [a, d]	152	11.84 (6.71–16.98) [b, d]	153	16.34 (10.48–22.20) [a, d]	153	1.96 (0.00–4.16) [a, c, d]	153	17.65 (11.61–23.69) [b, d, e]	152	8.55 (4.11–13.00) [a]	67.76 (60.33–75.19) [d]
EL	31	25.81 (10.40–41.21) [b, d, e, f]	9	7R/2S -	30	30.00 (13.60–46.40) [d, e]	0	- -	26	34.62 (16.33–52.90) [e, f]	0	- -	- -
IT	69	26.09 (15.73–36.45) [e, f]	69	24.64 (14.47–34.80) [e]	69	31.88 (20.89–42.88) [e]	69	14.49 (6.19–22.80) [e]	69	28.99 (18.28–39.69) [e, f]	69	28.99 (18.28–39.69) [d]	63.77 (52.43–75.11) [d, e, f]
NL	1461	10.81 (9.22–12.41) [a]	1380	3.77 (2.76–4.77) [a, c]	1457	4.94 (3.83–6.05) [b, c]	81	3.70 (0.00–7.82) [a, c, f]	1459	10.21 (8.66–11.77) [a]	1380[d]	2.25[d] (1.46–3.03) -	81.30[d] (79.25-83.36) -
PT	27	48.15 (29.30-66.99) [e]	32	31.25 (15.19-47.31) [e]	31	29.03 (13.05-45.01) [d, e]	30	10.00 (0.00-20.74) [b, d, e, f]	31	32.26 (15.80-48.71) [e, f]	25	24.00 (7.26-40.74) [c, d, e]	32.00 (13.71–50.29) [g, h]

Table 3 (continuation) - Percentage of resistance in *E. coli* by antimicrobial and country in 2012–2013

Country[a]	AMC		3GC		FLU		CN		SXT		Combined resistance		
	n	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% MDR (95% CI)[b] [Stat. Dif.][c]**	**% FullS (95% CI)[b] [Stat. Dif.][c]**
RS	3	2R/1S -	2	1R/1S -	3	0R/3S -	3	0R/3S -	3	1R/2S -	2	1MDR -	1FullS -
ES	60	31.67 (19.90–43.44) [e, f]	52	21.15 (10.05–32.25) [d, e]	61	29.51 (18.06–40.95) [e]	46	15.22 (4.84–25.60) [b, e]	60	26.67 (15.48–37.86) [e, f]	37	29.73 (15.00–44.46) [d, e]	43.24 (27.28–59.21) [e, g]
SE	2091	6.98 (5.89–8.07) [g]	2082	0 - [f]	2091	1.05 (0.61–1.49) [f]	2091	0.19 (0.00–0.38) [g]	2091	4.97 (4.04–5.91) [c]	2082	0.24 (0.03–0.45) [f]	90.2 (88.92–91.48) [b, h]
CH	133	10.53 (5.31–15.74) [a, g]	133	8.27 (3.59–12.95) [b, c]	132	13.64 (7.78–19.49) [a, d]	132	6.82 (2.52–11.12) [b, d, e, f]	131	13.74 (7.85–19.64) [a, d, g]	130	10.00 (4.84–15.16) [a, e]	83.08 (76.63–89.52) [a, c]
UK	143	21.68 (14.92–28.43) [b, f]	143	20.98 (14.31–27.65) [d, e]	143	11.89 (6.58–17.19) [a]	92	6.52 (1.48–11.57) [a, e, f]	142	21.13 (14.41–27.84) [b, f, g]	89	15.56 (8.07–23.04) [a, e]	67.78 (58.12–77.43) [a, d, f]

Legend: AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; FLU, fluoroquinolones; CN, gentamicin; SXT, trimethoprim/sulfamethoxazole; MDR, multidrug-resistant; FullS, fully-susceptible;

[a]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;

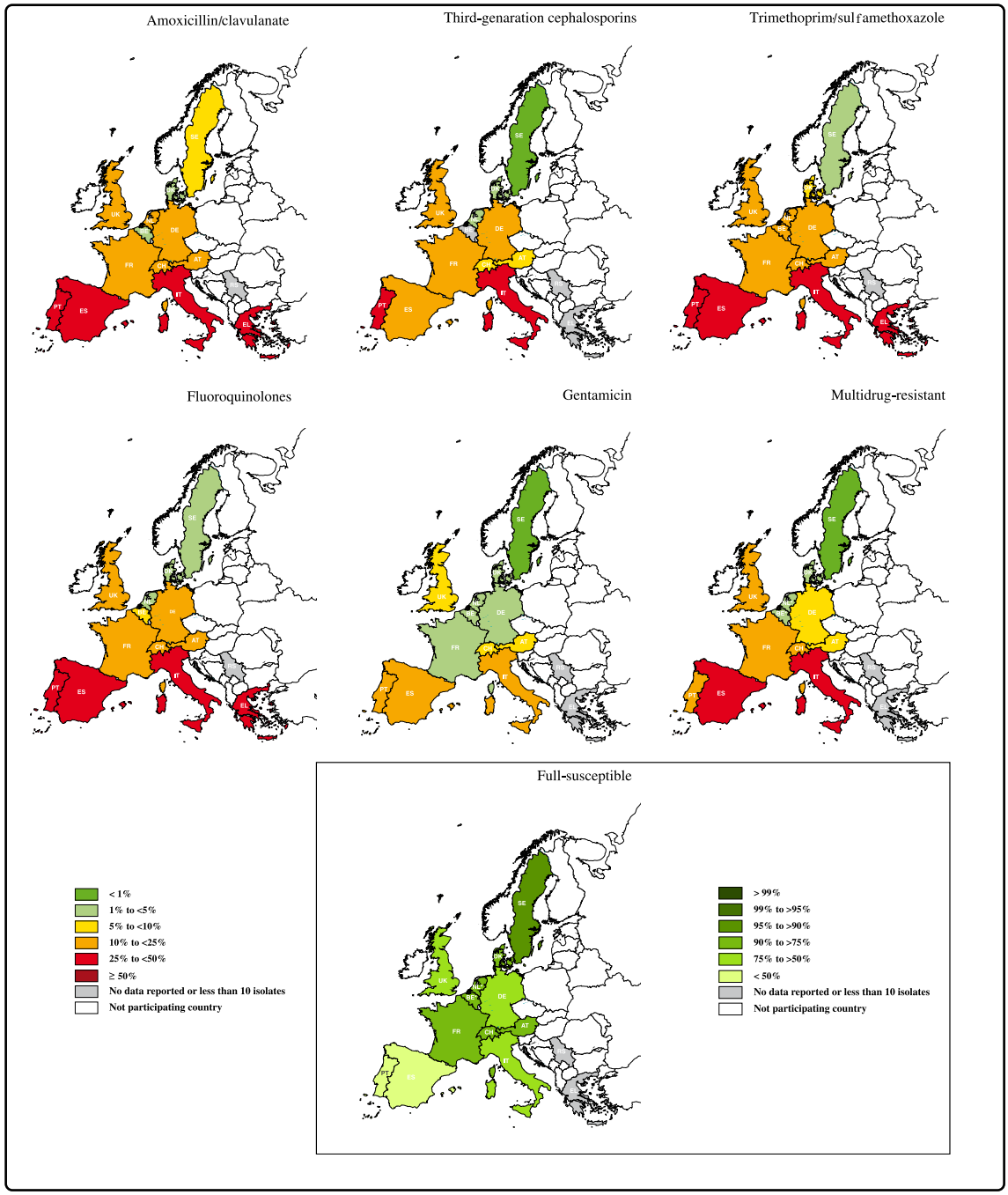
[b]95% CI, 95% Confidence interval;

[c]Stat. Dif., Statistical significant differences. Countries with no statistical difference are marked with the same letter. Countries were compared by Fisher exact test with an alpha value of 0.05. Countries with less than ten tested isolates were not compared. Regarding MDR and FullS, only countries tested for all the considered antimicrobials were compared;

[d]MDR and FullS percentages do not include resistance to 3GC for Belgium and resistance to CN for the Netherlands;

n, Total number of *E. coli* tested for the considered antimicrobial category.

Figure 2 - Percentage (%) of *E. coli* antimicrobial resistance by antimicrobial and country in the years 2012–2013



Legend: 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.
Multidrug resistance considering combined resistance to three or more of the following antimicrobial categories: AMC, 3GC, FLU, CN and SXT. Full-susceptibility (FullS) was defined as an isolate being susceptible for all the above-mentioned categories of antimicrobials.
Regarding multidrug resistance and full-susceptibility frequencies, countries marked by asterisk: 3GC was not included for Belgium and CN for the Netherlands. Thus, these frequencies may be underestimated when compared with the remaining countries.

Table 4 - Percentage of resistance in *Proteus* spp. by antimicrobial and country in 2012-2013

Country[a]	AMC		3GC		FLU		CN		SXT		Combined resistance		
	n	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% MDR (95% CI)[b] [Stat. Dif.][c]**	**% FullS (95% CI)[b] [Stat. Dif.][c]**
AT	29	10.34 (0.00–21.43) [a, b, c]	29	0 - [a, b]	29	17.24 (3.49–30.99) [a, b, c]	29	6.90 (0.00–16.12) [a, b]	29	37.93 (20.27–55.59) [a, b]	29	6.90 (0.00–16.12) [a]	55.17 (37.07–73.27) [a, b]
BE	143	2.10 (0.00–4.45) [a, g]	0	- - -	135	28.15 (20.56–35.73) [a, d]	155	9.68 (5.02–14.33) [a]	154	35.06 (27.53–42.60) [a, b]	125d	4.80[d] (1.05-8.55) -	57.60[d] (48.94–66.26) -
DK	31	0 - [a, d]	31	0 - [a, b]	31	0 - [e, f]	31	0 - [a, b]	31	6.45 (0.00–15.10) [c]	31	0 - [a, b]	93.55 (44.41–84.90) [c]
FR	215	7.44 (3.93–10.95) [b, d]	211	6.64 (3.28-9.99) [a]	212	17,92 (12.76-23.09) [b, g]	214	9.81 (5.83-13.80) [a]	216	27.78 (21.80-33.75) [a]	204	10.29 (6.12-14.46) [a]	66.67 (60.20-73.14) [a]
DE	10	0 - [a, b, f]	10	10.00 (0.00–28.59) [a, b, c]	10	50.00 (19.01–80.99) [a, d]	10	0 - [a, b, c]	10	20.00 (0.00–44.79) [a, c, d]	10	0 - [a, b]	50.00 (19.01–80.99) [a, d]
EL	8	1R/7S -	0	- -	8	4R/4S -	0	- -	7	4R/3R -	0	- -	- -
IT	12	0 - [a, b, c]	12	8.33 (0.00–23.97) [a, b, c]	12	41.67 (13.77–69.56) [a, d, g]	12	8.33 (0.00–23.97) [a, b, c]	12	66.67 (39.99–93.34) [b, e]	12	8.33 (0.00–23.97) [a, b, c]	16.67 (0.00–37.75) [d]
NL	261	6.13 (3.22–9.04) [a, b, e]	244	2.87 (0.77–4.96) [a]	260	8.85 (5.39–12.30) [c, e, h]	17	11.76 (0.00–27.08) [a, c]	260	27.31 (21.89–32.72) [a]	243d	3.29[d] (1.05–5.54) -	69.96[d] (64.19–75.72) -
PT	14	50.00 (23.81–76.19) [e]	15	33.33 (9.48–57.19) [c, d]	15	40.00 (15.21–64.79) [a, g, i]	15	33.33 (9.48–57.19) [c]	15	46.67 (21.42–71.91) [a, e]	14	42.86 (16.93–68.78) [c]	35.71 (10.61–60.81) [b, d, e]
RS	1	0R/1S -	1	1R/0S -	1	0R/1S -	1	0R/1S -	0	- -	0	- -	- -
ES	15	26.67 (4.29–49.05) [c, e, f]	13	15.38 (0.00–35.00) [a, d]	15	53.33 (28.09–78.58) [d, i]	9	2R/7S - -	15	53.33 (28.09–78.58) [b, d, e]	7	2MDR - -	4FullS - -

Table 4 (continuation)- Percentage of resistance in *Proteus* spp. by antimicrobial and country in 2012-2013

Country[a]	AMC		3GC		FLU		CN		SXT		Combined resistance		
	n	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% R (95% CI)[b] [Stat. Dif.][c]**	***n***	**% MDR (95% CI)[b] [Stat. Dif.][c]**	**% FullS (95% CI)[b] [Stat. Dif.][c]**
SE	170	2.35 (0.07–4.63) [a, f, g]	169	0 - [b, e]	170	0.59 (0.00–1.74) [f]	170	0.59 (0.00–1.74) [b]	170	7.06 (3.21–10.91) [c]	169	0 - [b]	91.12 (86.84–95.41) [c]
CH	17	0 - [a, b, f]	17	0 - [a, e]	17	23.53 (3.37–43.69) [a, g, h, l, j]	17	5.88 (0.00–17.07) [a, b, c]	17	35.29 (12.58–58.01) [a, e]	17	0 - [a, b]	64.71 (41.99–87.42) [a, e]
UK	16	12.50 (0.00–28.70) [a, b, f]	16	0 - [a, e]	16	0 - [b, e, f, j]	11	0 - [a, b, c]	15	33.33 (9.48–57.19) [a, e]	10	0 - [a, b]	70.00 (41.60–98.40) [a, c, e]

Legend: AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; FLU, fluoroquinolones; CN, gentamicin; SXT, trimethoprim/sulfamethoxazole; MDR, multidrug-resistant; FullS, fully-susceptible;

[a]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;

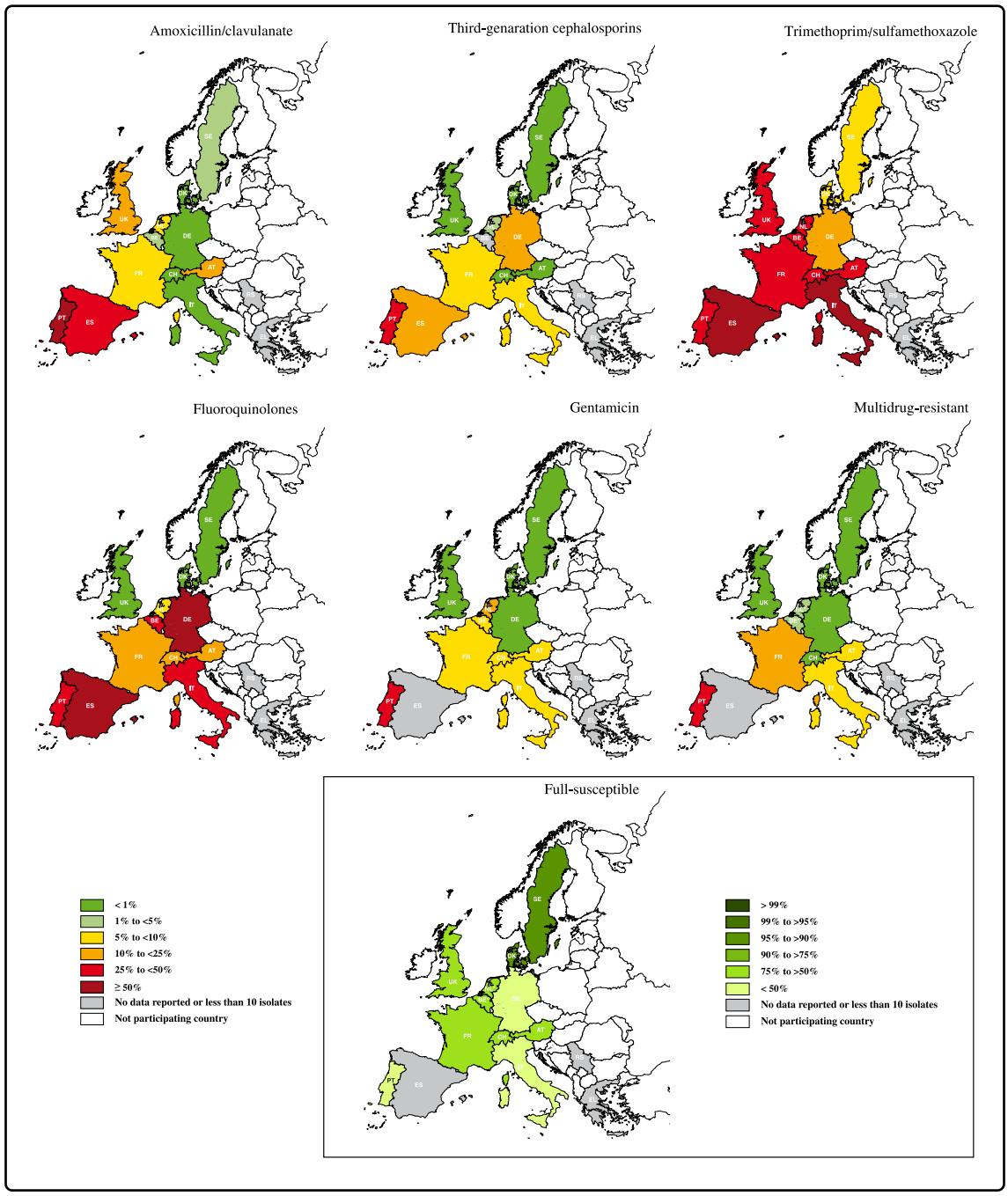
[b]95% CI,95 % Confidence interval;

[c]Stat. Dif., Statistical significant differences. Countries with no statistical difference are marked with the same letter. Countries were compared by Fisher exact test with an alpha value of 0.05. Countries with less than ten tested isolates were not compared. Regarding MDR and FullS, only countries tested for all the considered antimicrobials were compared;

[d]MDR and FullS percentages do not include resistance to 3GC for Belgium and resistance to CN for the Netherlands;

n, Total number of *Proteus* spp. tested for the considered antimicrobial category.

Figure 3 - Percentage (%) of *Proteus* spp. antimicrobial resistance by antimicrobial and country in the years 2012–2013



Legend: 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.
Multidrug resistance considering combined resistance to three or more of the following antimicrobial categories: AMC, 3GC, FLU, CN and SXT. Full-susceptibility was defined as an isolate being susceptible for all the above-mentioned categories of antimicrobials.
Regarding multidrug resistance and full-susceptibility frequencies, countries marked by asterisk: 3GC was not included for Belgium and CN for the Netherlands. Thus, these frequencies may be underestimated when compared with the remaining countries.

resistance frequency ($P < 0.05$) when compared with all countries except for Spain, Italy and Greece (Table 3). Less than 15% of *Proteus* spp. were resistant to AMC in all countries with exception of Portugal (50%) and Spain (26.67%) (Table 4).

E. coli resistance to 3GC had a similar distribution to what was seen for AMC (Figure 2). The highest 3GC-resistance frequencies were found in Southern countries, namely Portugal (31.25%), Italy (24.64%) and Spain (21.15%) (Table 3). *Proteus* spp. 3GC-resistance was lower than 5% in Austria, Denmark, Sweden, Switzerland, United Kingdom and the Netherlands, whereas Portugal (33.33%) and Spain (15.38%) were the countries with the highest resistance levels (Table 4).

SXT-resistance in Southern countries was higher than 25 and 45% for *E. coli* and *Proteus* spp., respectively (Tables 3 and 4; Figures 2 and 3). Sweden and Denmark had the lowest SXT-resistance values (lower than 9%). The remaining included countries had frequencies ranging between 10.21-21.13% and 20–37.93% in *E. coli* and *Proteus* spp., respectively (Tables 3 and 4).

E. coli FLU-resistance was higher in the Southern countries and ranged from 29.03% in Portugal to 31.88% in Italy (Table 3). Concerning *Proteus* spp., Spain and Germany had around 50% FLU-resistance, followed by Italy and Portugal with around 40% (Table 4). Sweden, Denmark, Belgium and the Netherlands had less than 10% FLU-resistant *E. coli*. Denmark, Sweden, United Kingdom and the Netherlands had less than 10% FLU-resistant *Proteus* spp. (Figures 2 and 3).

Overall, *E. coli* CN-resistance was lower than 16% (Table 3). Regarding resistance to CN in *Proteus* spp. the same resistance frequency occurred with the exception of Portugal where a higher resistance frequency was recorded (33.33%) (Table 4).

E. coli from Portugal, Spain and Italy, and *Proteus* spp. from Portugal showed the highest frequencies of MDR (Tables 3 and 4). As expected, Portugal was one of the countries with lowest FullS, both in *E. coli* (32.00%) and *Proteus* spp. (35.71%). Italy had even lower FullS in *Proteus* spp. (16.67%). Most of the remaining countries had MDR levels lower than 10%, with the exception of MDR *E. coli* from United Kingdom (15.56%) and France (11%). The highest *E. coli* and *Proteus* spp. FullS frequencies were found in Denmark and Sweden (Tables 3 and 4).

Due to the limited number of *Staphylococcus* spp. isolates available, the percentage of resistance to antimicrobials was determined on fewer countries for this group of bacteria (Tables 5 and 6). In most countries, *Staphylococcus pseudintermedius* was the most frequently isolated followed by coagulase negative staphylococci (CoNS) (Table 5). In general, the overall antimicrobial resistance levels in Southern countries were higher than in Northern countries (Figure 4) as seen in Gram-negative bacteria (Figures 2 and 3).

Table 5 - *Staphylococcus* spp. and methicillin-resistance by country in 2012–2013

Country[a]	Staphylococci species by country						Methicillin-resistance within each group					
							MRSA		MRSP		MRCoNS	
	N	SA	SP	CoPS	CoNS	SPP	*n* tested	R % (95% CI)[b]	*n* tested	R % (95% CI)[b]	*n* tested	R % (95% CI)[b]
AT	78	7.69 (1.78–13.61)	19.23 (10.48–27.98)	6.41 (0.97–11.85)	56.41 (45.41–67.41)	10.26 (3.52–16.99)	5	1R/4S -	15	33.33 (9.48–57.19)	43	27.91 (14.50–41.31)
BE	122	14.75 (8.46–21.05)	52.46 (43.60–61.32)	0 -	32.79 (24.46–41.12)	0 -	18	5.56 (0–16.14)	64	12.50 (4.40–20.60)	40	25.0 (11.58–38.42)
DK	52	1.92 (0–5.66)	53.85 (40.30–67.40)	1.92 (0–5.66)	28.85 (16.53–41.16)	13.46 (4.18–22.74)	1	1S -	27	0 -	15	46.67 (21.42–71.91)
FR	242	8.26 (4.80–11.73)	72.73 (67.12–78.34)	0 -	19.01 (14.06–23.95)	0 -	20	40.0 (18.53–61.47)	0	- -	46	17.39 (6.44–28.34)
DE	64	4.69 (0–9.87)	50.0 (37.75–62.25)	0 -	39.06 (27.11–51.02)	6.25 (0.32–12.18)	0	- -	0	- -	0	- -
EL	10	0 -	10.0 (0–28.59)	0 -	0 -	90 (71.41–100)	-	- -	1	1R -	-	- -
IT	19	0 -	94.74 (84.70–100)	0 -	5.26 (0–15.30)	0 -	-	- -	18	50.0 (26.90–73.10)	0	- -
NL	365	4.93 (1.12–2.71)	47.95 (42.82–53.07)	0 -	47.12 (42.0–52.24)	0 -	0	- -	174	10.92 (6.29–15.55)	172	0.58 (0–1.72)
PT	7	1SA -	4SP -	0CoPS -	0CoNS -	2SPP -	1	1R -	4	2R/2S -	0	- -
RS	0	- -	- -	- -	- -	- -	-	- -	-	- -	-	- -
ES	13	0 -	0 -	15.38 (0–35.0)	46.15 (19.05–73.25)	38.46 (12.02–64.91)	-	- -	-	- -	0	- -
SE	325	8.62 (5.56–11.67)	53.54 (48.12–58.96)	2.46 (0.78–4.15)	32.31 (27.22–37.39)	3.08 (1.20–4.95)	28	0 -	174	1.15 (0–2.73)	105	4.76 (0.69–8.84)

Table 5 (continuation)- *Staphylococcus* spp. and methicillin-resistance by country in 2012–2013

Country[a]	Staphylococci species by country						Methicillin-resistance within each group					
	N	SA	SP	CoPS	CoNS	SPP	MRSA		MRSP		MRCoNS	
		% (95% CI)[b]	% (95% CI)[b]	% (95% CI)[b]	% (95% CI)[b]	% (95% CI)[b]	*n* tested	R % (95% CI)[b]	*n* tested	R % (95% CI)[b]	*n* tested	R % (95% CI)[b]
CH	46	4.35 (0–10.24)	52.17 (37.74–66.61)	4.35 (0–10.24)	34.78 (21.02–48.55)	4.35 (0–10.24)	2	0R/2S -	20	10.00 (0–23.15)	15	66.67 (42.81–90.52)
UK	32	12.50 (1.04–23.96)	53.13 (35.84–70.41)	6.25 (0–14.64)	18.75 (5.23–32.27)	9.38 (0–19.47)	3	1R/2S -	12	8.33 (0–23.97)	3	1R/2S -

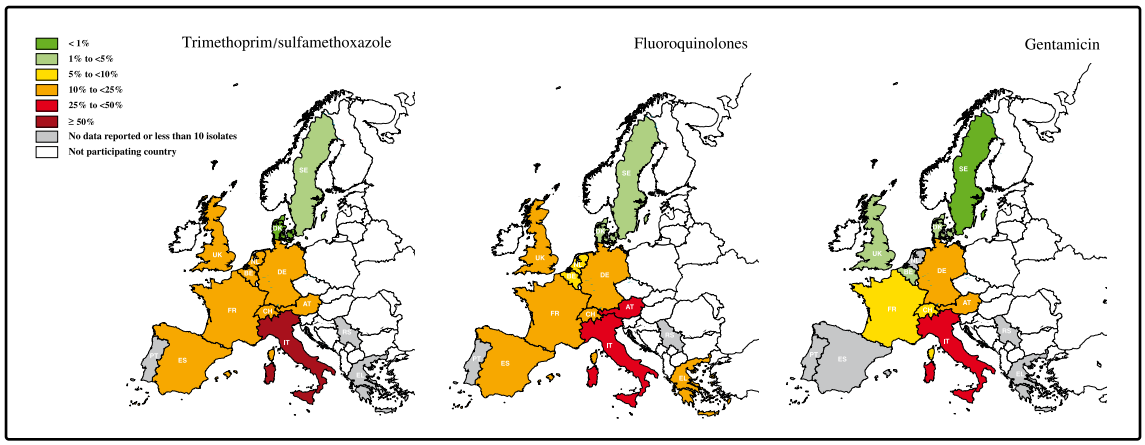
Legend: Staphylococci identification varied according to the country. Some countries identified staphylococci to species level, others to genus level and others included data on the coagulase test. Thus, the staphylococci results were grouped as follows: 1. *Staphylococcus aureus* (SA); 2. *Staphylococcus pseudintermedius* (SP); 3. coagulase positive staphylococci (CoPS), 4. coagulase negative staphylococci (CoNS) and 5. other staphylococci (SPP). Group 3 includes staphylococci identified only as CoPS or staphylococci species known to be coagulase positive other than SA and SP. Group 4 includes staphylococci identified only as CoNS or staphylococci species known to be coagulase negative. Group 5 includes staphylococci identified as *Staphylococcus* spp.;
MRSA methicillin-resistant *Staphylococcus aureus*, MRSP methicillin-resistant *Staphylococcus pseudintermedius*, MRCoNS methicillin-resistant coagulase negative staphylococci;
[a]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;
[b]95 % CI, 95 % Confidence interval;
N, Total number of staphylococci;
n tested, number of staphylococci tested for methicillin-resistance within each group.

Table 6 - Percentage of resistance in *Staphylococcus* spp. by antimicrobial and country in 2012–2013

Country[a]	FLU		CN		SXT	
	n	% R (95% CI)[b] [Stat. Dif.][c]	*n*	% R (95% CI)[b] [Stat. Dif.][c]	*n*	% R (95% CI)[b] [Stat. Dif.][c]
AT	78	26.92 (17.08–36.77) [a, b]	78	19.23 (10.48–27.98) [a]	78	20.51 (11.55–29.47) [a, b]
BE	116	7.76 (2.89–12.63) [c, d]	107	3.74 (0.14–7.33) [b]	122	13.11 (7.12–19.10) [a, b, c]
DK	51	1.96 (0.00–5.77) [c, e, f]	51	3.92 (0.00–9.25) [b]	51	0 - [d]
FR	238	23.53 (18.14–28.92) [a, g]	237	5.06 (2.27–7.85) [b]	242	11.57 (7.54–15.60) [a, c]
DE	55	18.18 (7.99–28.38) [a, d, g]	55	10.91 (2.67–19.15) [a, b]	55	23.64 (12.41–34.86) [b]
EL	10	20.00 (0.00–44.79) [a, d, e, g, h]	0	- - -	9	1R/8S - -
IT	19	42.11 (19.90–64.31) [a]	19	26.32 (6.52–46.12) [a]	19	63.16 (41.47–84.85) [e]
NL	364	6.59 (4.04–9.14) [c, i]	9	1R/8S - -	365	11.51 (8.23–14.78) [c]
PT	6	3R/3S - -	7	2R/5S - -	7	1R/6S - -
RS	0	- - -	0	- - -	0	- - -
ES	13	15.38 (0.00-35.00) [a, d, e, h, i]	8	0R/8S - -	11	18.18 (0.00-40.97) [a, b, c]
SE	325	1.54 (0.20–2.88) [f]	325	0 - [c]	325	2.77 (0.99–4.55) [d]
CH	41	24.39 (11.25–37.54) [a, b]	41	9.76 (0.67–18.84) [a, b]	41	19.51 (7.38–31.64) [a, b, c]
UK	31	12.90 (1.10–24.70) [b, c, e, g]	22	4.55 (0.00–13.25) [a, b, c]	30	16.67 (3.33–30.00) [a, b, c]

Legend: FLU, fluoroquinolones; CN, gentamicin; SXT, trimethoprim/sulfamethoxazole;
[a]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;
[b]95% CI,95 % Confidence interval;
[c]Stat. Dif., Statistical significant differences. Countries with no statistical difference are marked with the same letter. Countries were compared by Fisher exact test with an alpha value of 0.05. Countries with less than ten tested isolates were not compared;
n, Total number of Staphylococcus spp. tested for the considered antimicrobial category

Figure 4 - Percentage (%) of *Staphylococcus* spp. antimicrobial resistance by antimicrobial and country in the years 2012–2013



Legend: 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

Besides the limited number of staphylococci, methicillin-resistance results were also limited due to the identification only to genus level or lack of testing the appropriate antimicrobial surrogate. Denmark and Sweden showed the lowest *S. pseudintermedius* methicillin-resistance (MRSP) (0 and 1.15%, respectively). The remaining countries had MRSP frequencies higher than 8%, attaining 50% in Italy. Methicillin-resistance was also high in CoNS (Table 5).

Staphylococci SXT-resistance ranged from 2.77 to 63.16% and showed similar geographical distribution to Gram-negative bacteria (Figure 4). Among the participating countries, Staphylococci FLU-resistance frequencies were higher in Italy (42.11%) and again lower in Sweden (1.54%) and Denmark (1.96%), with the remaining countries varying between 6.59 and 26.92% (Table 6). Italy, Austria, Germany and Switzerland CN-resistant staphylococci frequencies ranged between 26.32 and 9.76% while the remaining countries had less than 6% (Table 6).

Regarding resistance temporal trends, most countries had no significant changes in *E. coli* resistance over the time periods considered (Table 7). Belgium showed a significant decrease in *E. coli* resistance to all antimicrobials and an increase in fully-susceptible isolates. Denmark (AMC, FLU, SXT), France (3GC, FLU), the Netherlands (3GC, FLU, SXT, MDR) and Sweden (CN, MDR) had also significant decreases in *E. coli* resistance over time (Table 7). However, the Netherlands (AMC) and Switzerland (CN) had a significant increase in *E. coli* resistance (Table 7). A rising trend was also detected in *Proteus* spp. FLU-resistance from Belgium (Table 8).

Table 7 - Temporal trends of antimicrobial resistance in *E. coli* by country

Country[a] (Years)	AMC	3GC	FLU	CN	SXT	MDR	FullS
	OR[b] (95% CI)[c] *P* value	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**
BE (2010–13)	**0.787 (0.646–0.960) 0.0180**	- - -	**0.749 (0.635–0.882) 0.0006**	**0.677 (0.507–0.904) 0.0081**	**0.796 (0.695–0.912) 0.0010**	**0.529[d] (0.393–0.712) <0.0001**	**1.275[d] (1.127–1.442) 0.0001**
DK (2008–13)	**0.698 (0.500–0.976) 0.0357**	0.869 (0.646–1.169) 0.3533	**0.742 (0.565–0.976) 0.0325**	0.926 (0.620–1.384) 0.7086	**0.793 (0.642–0.980) 0.0316**	0.874 (0.615–1.242) 0.4528	**1.396 (1.156–1.684) 0.0005**
FR (2010–13)	0.885 (0.780–1.005) 0.0606	**0.859 (0.749–0.987) 0.0314**	**0.822 (0.727–0.928) 0.0016**	0.938 (0.734–1.200) 0.6121	0.960 (0.853–1.080) 0.4997	0.901 (0.782–1.037) 0.1448	**1.112 (1.002–1.233) 0.0456**
DE (2009–13)	1.029 (0.779–1.358) 0.8424	1.076 (0.805–1.438) 0.6211	1.185 (0.912–1.540) 0.2037	0.856 (0.520–1.409) 0.5397	1.040 (0.831–1.302) 0.7317	1.111 (0.801–1.541) 0.5295	0.941 (0.780–1.136) 0.5281
EL[e] (2009–13)	1.534 (0.851–2.766) 0.1545	1.083 (0.586–2.003) 0.7992	0.924 (0.630–1.355) 0.6855	- - -	0.880 (0.596–1.301) 0.5229	- - -	- - -
IT (2009–2013)	1.175 (0.844–1.637 0.3391)	1.017 (0.749–1.383) 0.9127	0.828 (0.629–1.090) 0.1784	1.007 (0.700–1.449) 0.9686	0.769 (0.582–1.016) 0.0645	1.065 (0.761–1.490) 0.7147	1.248 (0.953–1.634) 0.1076
NL (2008–13)	**1.108 (1.026–1.197) 0.0088**	**0.465 (0.402–0.539) <0.0001**	**0.916 (0.841–0.999) 0.0464**	0.682 (0.327–1.422) 0.3071	**0.917 (0.859–0.978) 0.0083**	**0.380[d] (0.320–0.450) <0.0001**	**1.648[d] (1494.–1.818) <0.0001**
PT (2008–13)	1.139 (0.913–1.419) 0.2482	1.187 (0.945–1.492) 0.1411	1.029 (0.823–1.287) 0.8029	1.222 (0.899–1.660) 0.2010	1.087 (0.867–1.364) 0.4680	1.156 (0.898–1.488) 0.2601	0.797 (0.629–1.010) 0.0608
ES (2010–13)	1.372 (0.855–2.201) 0.1899	1.551 (0.857–2.808) 0.1474	0.801 (0.529–1214) 0.2965	0.859 (0.467–1.578) 0.6238	0.752 (0.489–1.156) 0.1939	1.237 (0.677–2.258) 0.4897	0.944 (0.570–1.564) 0.8234

Table 7 (continuation)- Temporal trends of antimicrobial resistance in *E. coli* by country

Country[a] (Years)	AMC	3GC	FLU	CN	SXT	MDR	FullS
	OR[b] (95% CI)[c] *P* value	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**	**OR[b] (95% CI)[c] *P* value**
SE (2008–13)	0.976 (0.915–1.041) 0.4569	- - -	0.980 (0.827–1.147) 0.8018	**0.700 (0.562–0.872) 0.0015**	0.961 (0.892–1.037) 0.3059	**0.697 (0.493–0.985) 0.0407**	1.035 (0.965–1.110) 0.3341
CH (2008–13)	1.143 (0.905–1.445) 0.2621	1.116 (0.861–1.447) 0.4067	1.007 (0.841–1.205) 0.9426	**1.493 (1.009–2.208) 0.0451**	1.080 (0.901–1.294) 0.4050	1.189 (0.920–1.536) 0.1863	1.025 (0.877–1.197) 0.7594
UK (2008–13)	1.075 (0.857–1.357) 0.5194	1.106 (0.873–1.400) 0.4041	0.945 (0.739–1.208) 0.6511	1.306 (0.792–2.155) 0.2952	0.972 (0.797–1.185) 0.7778	1.355 (0.954–1.925) 0.0892	1.154 (0.950–1.401) 0.1492

Legend: AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; FLU, fluoroquinolones; CN, gentamicin; SXT, trimethoprim/sulfamethoxazole; MDR, multidrug-resistant; FullS, fully-susceptible;

[a]BE, Belgium; DK, Denmark; FR, France; DE, Germany; EL, Greece; IT, Italy; NL, the Netherlands; PT, Portugal; ES, Spain; SE, Sweden; CH, Switzerland; UK, United Kingdom;

[b]OR, Odds ratio;

[c]95% CI, 95% Confidence interval;

[d]MDR and FullS temporal trends do not include resistance to 3GC for Belgium and CN for the Netherlands;

[e]Data regarding the years 2010 and 2012 were excluded from Greece resistance trends analysis since less than ten isolates were tested in those years;

Statistically significant trends are highlighted in bold.

Table 8 - Temporal trends of antimicrobial resistance in *Proteus* spp. by country

Country[a] (Years)	AMC OR[b] (95% CI)[c] *P* value	FLU OR[b] (95% CI)[c] *P* value	SXT OR[b] (95% CI)[c] *P* value
BE (2010–13)	0.627 (0.312–1.259) 0.1889	**1.292 (1.006–1.659)** **0.0450**	0.891 (0.726–1.092) 0.2649
FR (2010–13)	0.945 (0.625–1.431) 0.7908	1.004 (0.764–1.319) 0.9770	0.954 (0.756–1.205) 0.6944
NL (2008–13)	1.092 (0.860–1.387) 0.4709	0.970 (0.829–1.135) 0.7067	1.010 (0.903–1.129) 0.8660
SE (2008–13)	0.892 (0.657–1.210) 0.4620	0.884 (0.494–1.583) 0.6788	0.941 (0.759–1.165) 0.5756

Legend: AMC, amoxicillin/clavulanate; FLU, fluoroquinolones; SXT, trimethoprim/sulfamethoxazole;
[a]BE, Belgium; DK, Denmark; FR, France; NL, the Netherlands; SE, Sweden; CH, Switzerland;
[b]OR, Odds ratio;
[c]95% CI, 95% Confidence interval;
Statistically significant trends are highlighted in bold.

Discussion

Published data on antimicrobial resistance in bacteria isolated from companion animal UTIs over Europe is scarce (Weese et al., 2011) and the comparison between studies is impaired by the use of different inclusion criteria and different time periods. Moreover, UTI resistance frequencies are usually reported together with susceptibility data from other sites of infection (Normand et al., 2000a; Ruscher et al., 2009), combining different bacteria genera (Hall et al., 2013; Dorsch et al., 2015) and several countries (Meunier et al., 2004; Kroemer et al., 2014). These facts impair the establishment of a global epidemiological overview of UTI bacteria resistance in Europe. This is the first large study to analyse antimicrobial susceptibility data of canine and feline isolates from several European countries allowing an epidemiological overview of UTI resistance trends in Europe.

In accordance to previous studies (Litster et al., 2007b; Ball et al., 2008; Hall et al., 2013; Windahl et al., 2014; Dorsch et al., 2015), *E. coli* was the most frequently isolated bacteria in dogs and cats. *Enterococcus* spp. presented a significantly higher frequency in cats and *Proteus* spp. in dogs. While not compared in previous studies, this difference could be expected based on some published data focussed on cats (Litster et al., 2007b; Dorsch et al., 2015) and dogs (Féria et al., 2002) separately.

One of the most important findings from this study was the overall higher resistance frequencies found in the Southern countries (Italy, Greece, Portugal and Spain) when compared with the Northern countries (Denmark and Sweden). The lower frequency of antimicrobial resistance in Northern countries, such as Sweden, is likely a consequence of the tight regulations and surveillance on antimicrobial prescribing and resistance in companion animals. In light of the present results, such strategies could be useful in aiming the reduction of antimicrobial resistance in the Southern countries.

Resistance to β-lactams

Amoxicillin/clavulanate

Considering that AMC is one of the most used antimicrobials in animals, the levels of resistance detected in this study are worrisome, especially in the Southern countries. Previous published reports showed different frequencies of AMC-resistance in *E. coli* and in *Proteus* spp. that are likely due to the fact they report to different time frames and inclusion criteria (Féria et al., 2000; Féria et al., 2002; Lanz et al., 2003; Grobbel et al., 2007a; Pedersen et al., 2007; Windahl et al., 2014; Criel et al., 2015). In the absence of clinical data it is not possible to know if this resistance relates to uncomplicated or complicated UTI (Weese et al., 2011). Thus, these results need to be further investigated in order to establish whether AMC is a suitable empiric therapeutic choice for companion animals UTI in Southern Europe.

Third-generation cephalosporins

Southern countries had also higher levels of resistance to 3GCs. Although Greece was not included due to limited data, considering that seven out of the nine tested isolates were resistant to 3GCs, one can expect the prevalence of 3GC-resistance to be high. Previous studies in Portugal found a considerable lower 3GC-resistance value (1.4%) in *E. coli* from dogs in earlier years (Féria et al., 2000). In the present work, the lower Swedish results for 3GC-resistance in *E. coli* and *Proteus* spp. are in agreement with early studies (Hagman & Greko, 2005). Being of critical importance to humans (WHO, 2017a), prudent use of 3GC is of upmost importance.

Methicillin-resistance

The frequency of methicillin-resistant staphylococci, especially *S. pseudintermedius* and CoNS, varied considerably between countries and confirmed previous reports on a low MRSP prevalence in Scandinavia compared to elsewhere in Europe (Swedres-Svarm, 2016). Resistance to methicillin in coagulase-positive *Staphylococcus* (*S. aureus* and *S. pseudintermedius*) was detected in this study and is a great animal and public health concern (Guardabassi & Prescott, 2015). Currently, the recommended methods for the detection of methicillin-resistance in staphylococci are mainly phenotypic but in some circumstances the molecular detection of the *mecA* gene is clinically and epidemiologically necessary (CLSI, 2013a; CLSI, 2015; European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2013). This should be taken in consideration for the harmonisation of veterinary susceptibility testing in Europe.

Resistance to fluoroquinolones

In this study, high resistance frequencies towards the fluoroquinolones were found in *E. coli*, *Proteus* spp. and *Staphylococcus* spp. isolates in the southern European countries but also in

Proteus spp. from Germany, Belgium and Switzerland and *Staphylococcus* spp. from Austria, Switzerland and France. Several authors (Grobbel et al., 2007a; Oliveira, Dias & Pomba, 2014; Tramuta et al., 2014; Windahl et al., 2014) have reported lower FLU-resistance frequencies than the ones found in this study, especially regarding the Southern countries (Oliveira et al., 2014; Tramuta et al., 2014). The results of high resistance frequencies towards the fluoroquinolones found in this study are concerning because fluoroquinolones are considered a good first choice for pyelonephritis treatment and should otherwise be used as a second line antimicrobial (Weese et al., 2011).

Resistance to folate inhibitors and to aminoglycosides

In this study, resistance to SXT in Europe was high, especially in *E. coli* and *Proteus* spp.. The higher SXT-resistance found in *Proteus* spp., than in *E. coli* from several European countries, is consistent with other reports (Grobbel et al., 2007a; Criel et al., 2015; Grobbel et al., 2007b). Compared with previous studies, these results show a superior SXT-resistance in *E. coli* and *Proteus* spp. from Italy and Portugal (Féria et al., 2000; Tramuta et al., 2014) and *Staphylococcus* spp. from Belgium (Criel et al., 2015).

Also in agreement to previous studies, gentamicin was the antimicrobial with lower resistance in *E. coli*, *Proteus* spp. and *Staphylococcus* spp. all-over Europe (Lanz et al., 2003; Hagman et al., 2005; Grobbel et al., 2007a; Grobbel et al., 2007b; Pedersen et al., 2007; Tramuta et al., 2014; Windahl et al., 2014). Nevertheless, the distribution seemed to follow the same pattern, with increased resistance in Southern over Northern countries.

Multidrug resistance

Finally, MDR bacteria presented the worst scenario once again in *E. coli* from Southern countries and in *Proteus* spp. from Portugal. The emergence of MDR bacteria in companion animals has been previously described (Pomba et al., 2010; Wagner et al., 2014) and represents a great therapeutic challenge and public health concern. However, MDR/FullS frequencies are seldom reported and published data account for different antimicrobials, thus impairing any comparisons with the present results (Féria et al., 2000; Lanz et al., 2003; Hagman et al., 2005; Grobbel et al., 2007b; Windahl et al., 2014).

Trends in antimicrobial resistance

The surveillance of antimicrobial resistance is an important tool to guide the implementation of antimicrobial stewardship strategies. In this study, most countries had no significant changes in antimicrobial resistance over the time frame considered. Nevertheless, decreasing trends in antimicrobial resistance were found in *E. coli*. These encouraging trends were not detected in AMC and CN-resistance in *E. coli* from the Netherlands and Switzerland, respectively, where an increasing trend was observed. Although no changes over time were detected in *E. coli*

resistance against AMC and 3CGs in Portugal, the considerably lower resistance frequencies previously reported in earlier years (Féria et al., 2000), point to a possible increasing trend (Féria et al., 2002). The same may be the case for *E. coli* AMC-resistance in Germany and Switzerland (Lanz et al., 2003; Grobbel et al., 2007a).

Despite reporting clear trends such as the difference in resistance between Northern and Southern countries, data from this study should be interpreted with caution. Due to the retrospective nature of this study, data on clinical history such as the type of UTI and previous antimicrobial treatment were unavailable. Furthermore, the use of laboratory data may represent a bias towards resistance, since urine cultures from complicated cases tend to be requested more often than simple uncomplicated UTI (Ball et al., 2008; Hall et al., 2013). These limitations are not restricted to certain countries, and are therefore not likely to hamper comparison of data across borders. Given the limitations of retrospective studies, a veterinary European surveillance network gathering data prospectively on antimicrobial resistance, as well as, on clinical data is of the upmost importance to facilitate development of national evidence-based guidelines that take into consideration type of UTI, local regulations and patterns of antimicrobial resistance.

The use of different susceptibility testing methods and different clinical breakpoints is considered a major limitation. The lack of harmonisation became evident in this study when trying to compare 3GC and methicillin-resistance. Although it also happens in the well-established EARS network reports of resistance on bacteria from human invasive infections (ECDC, 2014), this limitation weakens the comparison of resistance between countries in the present and future surveillance studies. This harmonisation would allow future within and between countries resistance frequencies comparisons over time and would also provide relevant information on the impact of different antimicrobial usage policies. Thus, the authors agree that the harmonisation of methods and interpretative criteria in veterinary medicine should be a priority. The role of the new veterinary committee on antimicrobial susceptibility testing VetCAST (Veterinary Committee on Antimicrobial Susceptibility Testing [VetCAST], (2015) may be crucial in this harmonisation process. Despite these limitations, the results from this study provide relevant and updated information on the current antimicrobial resistance in UTI bacteria from companion animals in Europe. Similar studies should also be conducted regarding other types of infection to improve the awareness on the European distribution of antimicrobial resistance in companion animals. Ideally, monitoring of companion animal antimicrobial resistance should be implemented in Europe, as it is the case for food producing animals. Such surveillance would provide crucial information to promote the appropriate use of antimicrobials and therefore limit the spread of resistance.

Conclusions

This work brings new insights into the current scenario of the European antimicrobial resistance bacteria isolated from companion animals with UTI. An important finding from this study was the higher frequency of resistance in Southern European countries (Italy, Greece, Spain, Portugal) when compared to Northern European countries (Denmark, Sweden). Furthermore, there is an evident need to harmonise methods and interpretative criteria in veterinary medicine. Given the limitations of retrospective studies, an European surveillance network gathering data on antimicrobial resistance is of the upmost importance to facilitate the development of national evidence-based guidelines.

3.2 Increase in antimicrobial resistance and emergence of major international high-risk lineages in companion animals with urinary tract infection: 16 years' retrospective study.

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Marques, C.*, Belas, A., Franco, A., Aboim, C., Gama, L.T., Pomba, C. (2017). Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in cats and dogs with urinary tract infection: 16 years' retrospective study. *The Journal of Antimicrobial Chemotherapy,* 73, 377-384.

*The author performed the antimicrobial susceptibility testing, DNA extraction, bacterial identification and PCR assays of around 60% of the bacterial isolates. The author conducted the screening and sequence analysis of the tested antimicrobial resistance mechanisms of about 70% of bacterial isolates. The author conducted the MLST analysis. The author performed data and statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on co-authors and reviewers revisions.

Partial results were presented as,

Three Oral communications at the international congress 24th ECVIM-CA, 2014, Mainz, Germany; at the international congress 25th ECCMID, 2015, Copenhagen, Denmark; and at the national congress XII Congresso Hospital Veterinário Montenegro, 2016, Santa Maria da Feira, Portugal;

Three Poster communications at the international congress ISFM Congress 2015, 2015, Porto, Portugal; at the international congress 26th ECCMID, 2016, Amsterdam, The Netherlands; and at the national congress XII Congresso Hospital Veterinário Montenegro, Santa Maria da Feira, Portugal;

These works were distinguished with two awards, namely, "Best oral presentation" at the 24th ECVIM-CA given by the European Society of Veterinary Nephrology and Urology (ESVNU); and "Best oral communication" at the XII Congresso Hospital Veterinário Montenegro.

Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in dogs and cats with urinary tract infection: 16 year retrospective study

Cátia Marques[1], Adriana Belas[1], Andreia Franco[1], Catarina Aboim[1], Luís Telo Gama[1], Constança Pomba[1]

[1]Laboratory of Resistance to Antibiotics and Biocides, CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-UL), Lisbon, Portugal;
[2]Animal Genetic Resources, CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-UL),Lisbon, Portugal.

Abstract

Objectives: To evaluate temporal trends in antimicrobial resistance, over 16 years, in bacteria isolated from dogs and cats with urinary tract infection (UTI) and the clonal lineages of bacteria harbouring critical antimicrobial resistance mechanisms.

Methods: Antimicrobial susceptibility testing was conducted for 948 bacteria isolated from dogs and cats with UTI (1999–2014). Resistance mechanisms were detected by PCR, namely ESBL/AmpC in third-generation cephalosporin (3GC)-resistant *Escherichia coli* and *Proteus mirabilis*, *mecA* in methicillin-resistant staphylococci, and *aac(6')-Ieaph(2")-Ia* and *aph(2")-1d* in high-level gentamicin-resistant (HLGR) enterococci. Resistant bacteria were typed by MLST, and temporal trends in *E. coli* and Enterobacteriaceae antimicrobial resistance were determined by logistic regression.

Results: Enterobacteriaceae had a significant temporal increase in resistance to amoxicillin/clavulanate, 3GCs, trimethoprim/sulfamethoxazole, fluoroquinolones, gentamicin and tetracycline ($P < 0.001$). An increase in MDR was also detected ($P < 0.0001$). 3GC-resistance was mainly caused by the presence of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$ in *E. coli* and the presence of $bla_{CMY\text{-}2}$ in *P. mirabilis*. Two major 3GC-resistant *E. coli* clonal lineages were detected: O25b:H4-B2-ST131 and ST648. The *mecA* gene was detected in 9.2% ($n = 11/119$) of *Staphylococcus* spp., including MRSA clonal complex (CC) 5 ($n = 2$) and methicillin-resistant *Staphylococcus epidermidis* CC5 ($n = 4$). A temporal increase in MDR methicillin-resistant *Staphylococcus pseudintermedius* was detected ($P = 0.0069$). Some ampicillin-resistant and/or HLGR *Enterococcus* spp. were found to belong to hospital-adapted CCs, namely *Enterococcus faecalis* ST6-CC6 ($n = 1$) and *Enterococcus faecium* CC17 ($n = 8$).

Conclusions: The temporal increase in antimicrobial resistance and in MDR bacteria causing UTI in dogs and cats creates important therapeutic limitations in veterinary medicine. Furthermore, the detection of MDR high-risk clonal lineages raises public health concerns since companion animals with UTI may contribute to the spread of such bacteria.

Introduction

Urinary tract infections (UTIs) are frequently diagnosed in veterinary medicine (Hall et al., 2013) and may require antimicrobial treatment (Weese et al., 2011). Since antimicrobial resistance is known to change geographically and over time (European Centre for Disease Prevention and Control [ECDC], 2015), updated and long-term studies are critical to investigate the spread of antimicrobial resistance. *Escherichia coli* and *Proteus mirabilis* are the most frequently isolated Gram-negative bacteria from dogs and cats with UTI, while *Staphylococcus* spp. and *Enterococcus* spp. are the most common Gram-positive bacteria (Litster et al., 2007b; Hall et al., 2013). These bacteria, isolated from dogs and cats, may

harbour clinically and epidemiologically important resistance mechanisms of human and veterinary relevance such as ESBL (Féria et al., 2002; Ewers et al., 2010), cephalosporinases (AmpC) (Wagner et al., 2014), PBP2a (Couto et al., 2016) and high-level gentamicin-resistance (HLGR) bifunctional enzyme (Jackson et al., 2010). Moreover, the detection of MDR bacteria in dogs and cats is being increasingly reported (Wagner et al., 2014; Couto et al., 2016), posing a difficult veterinary therapeutic challenge and often requiring the use of antimicrobials critically important to humans (WHO, 2017a). 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, several studies have shown that dogs and cats may share uropathogenic bacteria with the remaining household members (Johnson et al., 2009). Therefore, the identification of the clonal lineages of bacteria isolated from dogs and cats with UTI, especially those harbouring important resistance mechanisms, is crucial to evaluate the extent to which dogs and cats with UTI may act as reservoirs for resistant bacteria.

The goal of this study was to determine the temporal trends of antimicrobial resistance of bacteria isolated from dogs and cats with UTI over 16 years and to characterise their major antimicrobial resistance mechanisms, namely ESBL and AmpC in *E. coli* and *P. mirabilis*, methicillin-resistance in *Staphylococcus* spp., and ampicillin and HLGR in *Enterococcus* spp. Furthermore, this study aimed to determine the clonal lineages of *E. coli*, *Staphylococcus* spp. and *Enterococcus* spp. harbouring such resistance genes and hence evaluate their potential public health relevance.

Materials and methods

Bacterial isolates

A total of 948 consecutive positive bacterial isolates from dogs and cats with UTI ($n = 869$), collected from 1999 to 2014 at the Laboratory of Antimicrobial Resistance from the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine/University of Lisbon and from several private practices in the Lisbon region were included in this study. Samples were collected by cystocentesis, catheterisation or free catch as part of the routine care of dogs and cats with UTI. UTI was diagnosed based on clinical and urine cytological findings together with the detection of significant bacteriuria by quantitative urine culture.

Bacteriological methods

Quantitative urine culture was performed. Briefly, 10 µL of urine was inoculated onto 5% sheep blood (bioMérieux, Marcy-l'Étoile, France) and MacConkey (Biokar Diagnostics, Allonne, France) agar plates. After incubation at 37 °C for 24–48 h under atmospheric conditions, colonies were quantified and scored as having significant bacteriuria according to the urine sample collection method as defined elsewhere (Bartges et al., 2004). Samples were included in the study regardless of the type of UTI.

Species identification was conducted by phenotypic tests (API, bioMérieux and BD™ BBL™ Crystal Gram Positive ID Kit, Becton Dickinson, MD, USA). At the time of collection, 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 the present study, stored isolates were recovered by streaking them onto 5% sheep blood agar. Whenever recovery was not possible, existing antimicrobial susceptibility results were used. *E. coli* (Doumith, Day, Hope, Wain & Woodford, 2012), *Klebsiella* spp. (Kovtunovych et al., 2003; Padmavathy et al., 2012), *Proteus* spp. (Stankowska, Kwinkowski & Kaca, 2008), *Enterococcus* spp. (Woodford, Egelton & Morrison, 1997) and *Staphylococcus* spp. (Couto et al., 2016) were confirmed by PCR and/or sequencing of 16S rRNA.

Susceptibility testing

Susceptibility testing was performed by the disc diffusion method according to CLSI guidelines, and *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used for quality control (CLSI, 2013b). The following antimicrobials were tested (Oxoid, Hampshire, UK): amoxicillin/clavulanate 30 µg, cefotaxime 30 µg, cefovecin 30 µg, cefoxitin 30 µg, ceftazidime 30 µg, ciprofloxacin 5 µg, enrofloxacin 5 µg, gentamicin 10 µg, high-level gentamicin 120 µg, oxacillin 1 µg, penicillin 10 U, tetracycline 30 µg and trimethoprim/sulfamethoxazole 25 µg. Veterinary CLSI breakpoints (CLSI, 2013a) were used for amoxicillin/clavulanate, cefoxitin, enrofloxacin, gentamicin, high-level gentamicin, oxacillin, penicillin, tetracycline and trimethoprim/sulfamethoxazole; human CLSI breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2016) were used for cefotaxime, ceftazidime and ciprofloxacin. Finally, cefovecin results were interpreted according to the manufacturer's breakpoints. Initial ESBL screening was conducted by a double-disc synergy test (CLSI, 2013b). Cefoxitin or oxacillin were used to predict methicillin-resistance in *Staphylococcus* spp. according to CLSI guidelines (CLSI, 2013a; CLSI, 2016).

DNA extraction, sample purification and sequencing

DNA extraction was conducted using a boiling method (Féria et al., 2002). 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/).

Antimicrobial resistance genes

Third-generation cephalosporin (3GC)-resistant *E. coli* and *P. mirabilis* were screened for the presence of ESBL $bla_{\text{CTX-M-type}}$ and AmpC $bla_{\text{CIT-type}}$, $bla_{\text{DHA-type}}$, $bla_{\text{MOX-type}}$, $bla_{\text{ACT-type}}$, $bla_{\text{FOX-type}}$

and *bla*$_{\text{MIR-type}}$ by PCR and sequencing (Pérez-Pérez & Hanson, 2002; Guessennd et al., 2008; Belas et al., 2014). 3GC-resistant Enterobacteriaceae without *bla*$_{\text{CTX-M-type}}$ or AmpC genes were further tested for the presence of *bla*$_{\text{TEM-type}}$ and *bla*$_{\text{SHV-type}}$ ESBL genes (Féria et al., 2002). *Staphylococcus* spp. were screened for the presence of the *mecA* gene (Couto et al., 2016) and HLGR *Enterococcus* spp. for the presence of *aac(6')-Ieaph(2")-Ia* and *aph(2")-1d* (Kao et al., 2000) genes by PCR. Negative and previously sequenced positive controls were included in all PCR reactions.

Clonal lineages of resistant isolates

ESBL- or AmpC-producing *E. coli* were tested for the clonal lineage O25b:H4-B2-ST131 by PCR (Doumith et al., 2012; Johnson et al., 2014). 3GC-resistant *E. coli* isolates not belonging to the O25b:H4-B2-ST131 clonal lineage were typed by MLST (Wirth et al., 2006). Methicillin-resistant *Staphylococcus* spp. were previously characterised by MLST, SCC*mec* and *spa* typing elsewhere (Couto et al., 2016). Ampicillin-resistant and/or HLGR *Enterococcus faecium* and *Enterococcus faecalis* were also typed by MLST (Homan et al., 2002; Ruiz-Garbajosa et al., 2006). eBURSTv.3 software (http://eburst.mlst.net/) was used to estimate the relationships between the isolate STs from this study and all MLST profiles known to date.

Statistical analysis

The SAS statistical software package for Windows v. 9.3 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Antimicrobial resistance frequencies were only calculated if at least 10 isolates were tested for a specific organism/antimicrobial combination and results were presented with the 95% CI. For statistical purposes, intermediate isolates were considered susceptible. An isolate was considered resistant to 3GCs when it was found to be resistant to at least one of the three 3GCs tested (cefotaxime, ceftazidime and cefovecin). Ciprofloxacin and enrofloxacin were used as markers of fluoroquinolone-resistance. An isolate was considered MDR when it was found to be fully-resistant to three or more antimicrobial categories. The antimicrobial categories were adapted from those proposed by other authors (Magiorakos et al., 2012) and varied according to the bacterial species considered (Table S1). When at least 10 isolates were tested per year, temporal trends of antimicrobial resistance were determined using an SAS LOGISTIC regression model with the year as a continuous variable and an α value of 0.05. Thus, temporal trends in antimicrobial resistance were determined for *E. coli* and for Enterobacteriaceae (including *E. coli*, *Proteus* spp., *Klebsiella* spp. and *Enterobacter* spp. as a group). When determining Enterobacteriaceae temporal trends, intrinsic resistance data was excluded from analysis. Furthermore, *P. mirabilis* and *Staphylococcus* spp. antimicrobial resistance were compared between two time periods: 1999–2006 and 2007–14. The Fisher's exact test was used for comparisons between groups with an α value of 0.05.

Results

From 1999 to 2014, 948 bacteria were isolated from 649 dogs and 220 cats with UTI. The majority of UTIs (91.1%, CI 89.2%–93.0%, *n* = 792/869) were caused by single organisms. Coinfections were most commonly caused by the combinations of *E. coli*/*Enterococcus* spp. (14.3%, CI 6.5%–22.1%, *n* = 11/77), *E. coli*/*P. mirabilis* (11.7%, CI 4.5%–18.9%, *n* = 9/77) and *E. coli*/*Streptococcus* spp. (10.4%, CI 3.6%–17.2%, *n* = 8/77).

Although *E. coli* (43.5%) was the most frequently isolated bacterium, *Proteus* spp. (16.4%), *Staphylococcus* spp. (13.2%) and *Enterococcus* spp. (7.0%) were also common (Table S2). The frequency of infection by *Proteus* spp. was significantly higher ($P < 0.0001$) in dogs and *Enterococcus* spp. in cats, respectively (Table S2). Overall, *Staphylococcus* spp. had similar frequencies in cats and dogs. However, *Staphylococcus pseudintermedius* was significantly more common in dogs ($P < 0.0001$), while cats were infected by a higher diversity of staphylococcal species, with *S. pseudintermedius*, *Staphylococcus felis* and *Staphylococcus* epidermidis being the most frequent (Table S2).

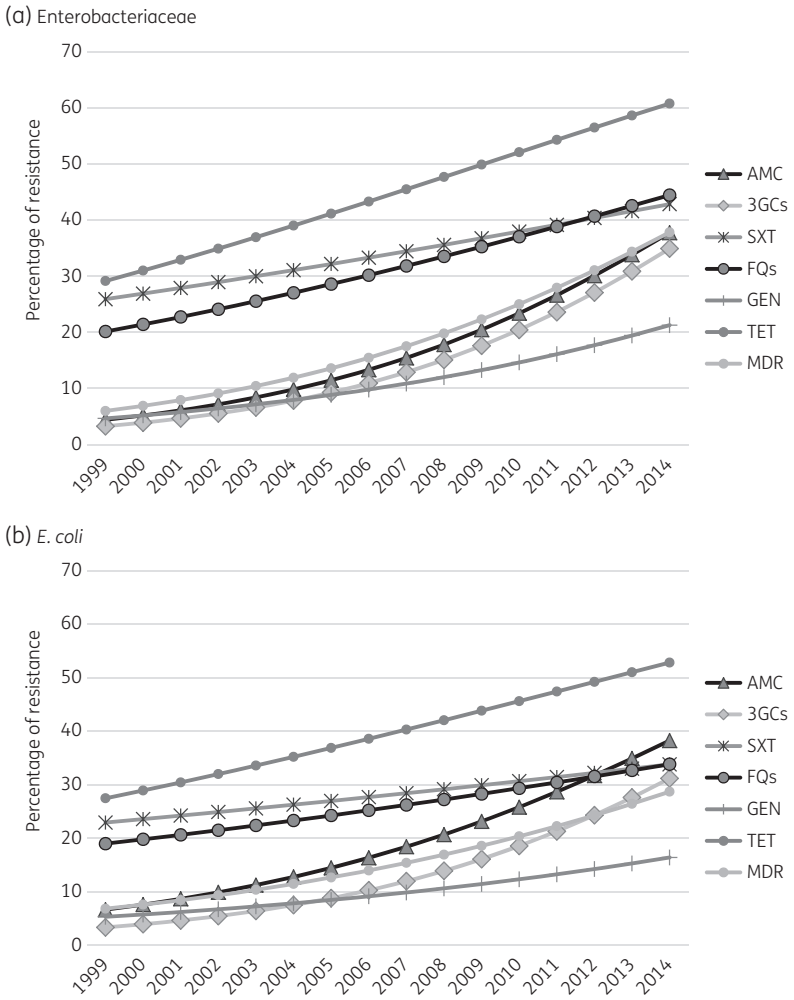
Enterobacteriaceae accounted for 66.1% (CI 63.1%–69.2%) of all isolated bacteria and showed a significant temporal increase in resistance to all tested antimicrobials (Table S3 and Figure 1). In 2012–14, resistance of Enterobacteriaceae to all the antimicrobials tested, except gentamicin, was >30% (Figure 1).

Considering *E. coli* and *Proteus* spp. alone, no significant change over time was detected in trimethoprim/sulfamethoxazole-resistance (Tables S3 and S4). Nevertheless, from 1999/2006 to 2007/2014, *E. coli* showed a 3-fold increase in amoxicillin/clavulanate-resistance and a 4-fold increase in 3GC-resistance. *Proteus* spp. had an even higher increase in resistance, showing a 5- and 9-fold increase in amoxicillin/clavulanate and 3GC-resistance, respectively (Table S4). *Proteus* spp. and *E. coli* also had a significant increase in gentamicin-resistance (Tables S3 and S4). Detection of MDR Enterobacteriaceae, *E. coli* and *Proteus* spp. increased significantly over time (Tables S3 and S4 and Figure 1). Most MDR Enterobacteriaceae (excluding *Enterobacter* spp.) were susceptible to at least one antimicrobial (Table S5). Gentamicin was the antimicrobial to which most MDR Enterobacteriaceae (excluding *Enterobacter* spp.) were susceptible (46.2%, CI 36.1%–56.4%). On the contrary, MDR Enterobacteriaceae were seldom susceptible to fluoroquinolones (9.7%, CI 3.7%–15.7%) (Table S5). Furthermore, among MDR *E. coli*, only 19.35% (CI 9.5%–29.2%) were susceptible to tetracycline.

A total of 33 *E. coli* and 9 *P. mirabilis* that were 3GC-resistant were recovered and screened for the presence of ESBL and AmpC genes. The first 3GC-resistant *E. coli* isolate was detected in 1999, yet none of the tested genes were detected. Another resistance mechanism may be involved. In total, only seven 3GC-resistant *E. coli* were negative for all tested genes, including $bla_{\text{TEM-type}}$ and $bla_{\text{SHV-type}}$ ESBLs. *E. coli* 3GC-resistance was mainly related to the presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ (Tables 1 and 2). The first CTX-M-producing *E. coli* was detected in

2004 and belonged to the O25b:H4-B2-ST131 clonal lineage (Table 1). Besides O25b:H4-B2-ST131, CTX-M-producing *E. coli* were frequently found to belong to clonal complex (CC) 23, including a novel ST described here (Table 1 and Figure S1). From 2010 onwards, an increase in 3GC-resistant *E. coli* ST648 harbouring bla_{CMY-2} was observed (Table 2). 3GC-resistant *P. mirabilis* was first detected in 2004. All isolates with this phenotype were bla_{CMY-2} positive, except one *P. mirabilis* from 2007 possessing bla_{DHA-1} (Table S6).

Figure 1 - Enterobacteriaceae and *E. coli* resistance trends over the 16 years of the study



Legend: Depiction of the temporal trends in resistance obtained by logistic regression over 16 years. (a) Enterobacteriaceae. There was a significant increase ($P < 0.05$) in antimicrobial resistance to all antimicrobials. (b) *E. coli*. There was a significant increase ($P < 0.05$) in antimicrobial resistance to all antimicrobials, except trimethoprim/sulfamethoxazole. AMC, amoxicillin/clavulanate; 3GCs, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline.

Regarding *Staphylococcus* spp., 9.2% (CI 4.0%–14.4%, $n = 11/119$) of *Staphylococcus* spp. were methicillin-resistant and were found to harbour the *mecA* gene. Overall only resistance to fluoroquinolones was significantly higher in the second time period (2007–14; $P = 0.0189$) (Table S4). However, if *S. pseudintermedius* are analysed alone, a significant increase in

Table 1 - CTX-M-producing *E. coli* clonal lineages

Isolate	Year	β-Lactamase	Clonal lineage	CC	Animal	MDR	AMC	3GCs	SXT	FQs	GEN	TET
FMV5825/04	2004	CTX-M-15	O25b:H4-B2-ST131	CC131	Dog	Yes	R	R	R	R	R	R
FMV521/07	2007	CTX-M-32	ST224	—	Cat	Likely	I	R	S	R	S	R
FMV1630/07	2007	CTX-M-15	unassigned ST[b]	CC23	Dog	Yes	I	R	R	R	S	R
FMV7261/07	2007	CTX-M-32	ST609	CC46	Dog	Yes	I	R	R	R	S	R
FMV635/08	2008	CTX-M-32	ST23	CC23	Cat	Yes	R	R	R	R	S	S
FMV2777/08	2008	CTX-M-15	O25b:H4-B2-ST131	CC131	Cat	Yes	R	R	S	R	R	R
FMV5827/08	2008	CTX-M-15	ST23	CC23	Dog	Yes	R	R	R	R	S	S
FMV1952/10[a]	2010	CTX-M-9	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV4479/13[a]	2013	CTX-M-15	ST533	—	Dog	Yes	R	R	R	R	S	S
FMV5338/13	2013	CTX-M-15	O25b:H4-B2-ST131	CC131	Dog	Yes	I	R	S	R	R	R
FMV58/2013	2013	CTX-M-1-type	O25b:H4-B2-ST131	CC131	Cat	Yes	S	R	R	R	S	R
FMV121/2014RE	2014	CTX-M-1-type	ST539	—	Dog	Yes	S	R	R	R	S	R

Legend: AMC, amoxicillin/clavulanate; 3GCs, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible;
[a]Also harbours bla_{CMY-2};
[b]Refer to Figure S1 to see the new ST allelic profile.

Table 2 - AmpC-producing *E. coli* clonal lineages

Isolate	Year	β-Lactamase	Clonal lineage	CC	Animal	MDR	AMC	3GCs	SXT	FQs	GEN	TET
FMV434/00	2000	CMY-2	ST1775	—	Dog	Yes	R	R	R	S	S	R
FMV1953/01	2001	CMY-2	ST57	CC350	Dog	Yes	R	R	R	I	I	R
FMV203/03	2003	CMY-2	ST405	CC405	Dog	Yes	R	R	S	R	S	R
FMV6346/05	2005	CMY-2	ST539	—	Cat	Yes	R	R	S	R	R	R
FMV3389/06	2006	CMY-2	ST354	CC354	Dog	Yes	R	R	R	R	R	R
FMV1952/10a	2010	CMY-2	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV25/2011	2011	CMY-2	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV29/2011	2011	CMY-2	ST648	CC648	Dog	Yes	R	R	S	R	R	R
FMV469/13	2013	CMY-2	ST648	CC648	Dog	Yes	R	R	R	R	R	R
FMV1389/13	2013	CMY-2	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV4479/13[a]	2013	CMY-2	ST533	—	Dog	Yes	R	R	R	R	S	S
FMV55/2013	2013	CMY-2	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV546/14	2014	CMY-2	ST648	CC648	Cat	Yes	R	R	R	R	R	R
FMV966/14	2014	CMY-2	ST648	CC648	Dog	Yes	R	R	R	R	S	R
FMV1549/14	2014	CMY-2	ST648	CC648	Cat	Yes	R	R	S	R	S	R
FMV43/2014	2014	CMY-2	ST648	CC648	Cat	Yes	R	R	S	R	S	R

Legend: AMC, amoxicillin/clavulanate; 3GCs, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible;
[a]Also harbours *bla*$_{CTX-M}$.

methicillin (14.8%, CI 1.4%–28.2%, in 2007–14; *P* = 0.0069) and gentamicin (17.9%, CI 3.7%–32.0%, in 2007–14; *P* = 0.0099) resistance was also detected. All MDR *Staphylococcus* spp. were associated with the presence of the *mecA* gene. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP; *n* = 4) and methicillin-resistant *S. epidermidis* (MRSE; *n* = 4) were the most common methicillin-resistant *Staphylococcus* species, followed by MRSA (*n* = 2) and methicillin-resistant *Staphylococcus lentus* (*n* = 1). Although uncommon, *S. epidermidis* showed a high frequency of methicillin-resistance (*n* = 4/6). MRSP showed resistance to all the tested antimicrobials, except two that were susceptible to tetracycline. Thus all MRSP were considered MDR. All methicillin-resistant *Staphylococcus* spp. but one were isolated from cats. The 11 methicillin-resistant *Staphylococcus* spp. found in this study were fully characterised elsewhere (Couto et al., 2016). Briefly, MRSP belonged to ST71-II-III (*n* = 3) and ST196-V (*n* = 1); MRSE belonged to CC5 (ST2-nt, ST20-nt, ST23-IV, ST35-nt) and MRSA to CC5 (ST5-t311-VI, ST105-t002-II).

Enterococcus spp. showed very high tetracycline (75.8%, CI 65.2%–86.5%, *n* = 47/62) and fluoroquinolone (56.4%, CI 44.1%–68.8%, *n* = 35/62) resistance. Ampicillin-resistance in *Enterococcus* spp. (12.1%, CI 4.2%–20.0%, *n* = 8/66) was lower, though if only *E. faecium* were considered, almost all were ampicillin-resistant (*n* = 8/9). HLGR was detected in 15.2% (CI 6.1%–24.4%, *n* = 9/59) of the tested *Enterococcus* spp. and was mostly found in *E. faecalis* harbouring *aac(6')-Ieaph(2")-Ia* (Table S7). HLGR *E. faecalis* belonged to known sequence types, including one isolate from ST6-CC6 (former CC2) (Table S7). All ampicillin-resistant and/or HLGR *E. faecium* belonged to CC17 (Table S7), including two novel STs (ST1282 and ST1283) identified in this study (Figure S2).

Discussion

As seen in other studies, *E. coli* was the most frequently isolated bacterium in dogs and cats with UTI (Litster et al., 2007b; Hall et al., 2013). Overall, Enterobacteriaceae caused more than half of the UTIs in dogs and cats; therefore, given that β-lactams are among the most important antimicrobials nowadays (WHO, 2017a), the great increase detected in this study in Enterobacteriaceae resistance to amoxicillin/clavulanate and 3GCs is worrisome (Figure 1 and Table S4).

3GCs are considered highest priority critically important antimicrobials for humans (WHO, 2017a), and resistance is frequently associated with the production of β-lactamases (ECDC, 2015). The increase in 3GC-resistance observed in this study was frequently associated with the presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ in *E. coli* and $bla_{\text{CMY-2}}$ in *P. mirabilis* (Tables 1 and 2 and Table S6).

ESBL CTX-M-15 is distributed worldwide in *E. coli* (Ewers et al., 2012). Moreover, *E. coli* O25b:H4-B2-ST131 is a widespread human uropathogenic clonal lineage that frequently harbours $bla_{\text{CTX-M-15}}$ (Nicolas-Chanoine et al., 2008) and has been previously described in dog

and cat faecal samples (as a commensal), (Johnson et al., 2009) and also causes UTI (Ewers et al., 2010). Therefore, the detection of *E. coli* O25b:H4-B2-ST131 in this study came as no surprise. Furthermore, *E. coli* CC23 has been described in humans with UTI in the community (Lau et al., 2008) and has also been shown to be a common CC among CTX-M-producing *E. coli* (Brisse et al., 2012).

This study shows an increase in the detection of CMY-2-producing *E. coli* and *Proteus* spp. over time. Although less frequently reported than $bla_{\text{CTX-M}}$ in previously published data (Aragón et al., 2008; Ewers et al., 2010), this increase in $bla_{\text{CMY-2}}$ should not be neglected since this enzyme shows stronger β-lactamase activity (Jacoby, 2009) and may in the future become more prevalent. Furthermore, $bla_{\text{CMY-2}}$-carrying Enterobacteriaceae may exhibit resistance to carbapenems in the absence of carbapenemases, owing to the presence of other resistance mechanisms such as porin deficiency (Chia et al., 2009), which further highlights their clinical relevance.

In the present study, the first CMY-2-producing *E. coli* was detected in 2000, yet it was from 2010 onwards that the *E. coli* CMY-2-producing ST648 clonal lineage was increasingly detected (Table 2). *E. coli* ST648 has been described in human infections harbouring several β-lactamases, such as ESBL and carbapenemases (Ewers et al., 2012; Pitout, 2012). Nevertheless, both in humans and companion animals, the ST648 *E. coli* clonal lineage has mostly been described harbouring $bla_{\text{CTX-M}}$ genes (Pitout, 2012; Ewers et al., 2014a). The significant increase in ST648 CMY-2-producing *E. coli* observed in this study in companion animals with UTI may point to the possible expansion of a $bla_{\text{CMY-2}}$-producing MDR ST648 clonal lineage, though more studies are needed to clarify the clonal relatedness between these isolates. However, a few studies have also found a high frequency of ST648 CMY-2-producing *E. coli* in faecal samples and specimens from infections of companion animals (Tamang et al., 2012; Liu et al., 2016). Although only detected once in this study, *E. coli* ST405 also belongs to a highly successful clonal lineage that causes human infection (Pitout, 2012). Dogs and cats with UTI are, therefore, shown in this study to be infected with 3GC-resistant *E. coli* belonging to clonal lineages of great importance to humans. Furthermore, CTX-M- and CMY-producing *E. coli* and *P. mirabilis* were also found to be MDR, thus increasing the relevance of these findings.

Additionally, Enterobacteriaceae from this study showed a significant increase in resistance to all the antimicrobials commonly used in the treatment of dogs and cats with UTI (Figure 1). Together with the significant increase in detection of MDR Enterobacteriaceae over time, these results point to growing therapeutic limitations in veterinary medicine that in the future may lead to an increasing need to prescribe antimicrobials originally intended for human use.

ESBL- and AmpC-producing *E. coli* presented MDR susceptibility phenotypes with limited therapeutic options (Tables 1 and 2). A study in humans showed that the use of amoxicillin/clavulanate for the treatment of UTIs caused by ESBL-producing *E. coli* may be

suitable if the isolate is fully-susceptible to this antimicrobial (Beytur et al., 2015). Although most ESBL-producing *E. coli* from this study displayed intermediate resistance to amoxicillin/clavulanate, similar studies should be conducted in veterinary medicine to evaluate its effectiveness in the treatment of MDR ESBL-producing Enterobacteriaceae.

MDR Enterobacteriaceae were more often susceptible to gentamicin; hence, although sometimes impractical, the use of this antimicrobial should be considered for the treatment of UTIs caused by MDR Enterobacteriaceae in dogs and cats (Table S5).

Staphylococcus spp. were the second most frequently isolated bacteria; however, the identified *Staphylococcus* species varied significantly between dogs and cats (Table S2). The high frequency of *S. felis* in cats with UTI is in agreement with a previous study conducted in Australia, in which the authors associated the presence of *S. felis* with clinical signs of lower urinary tract disease in cats (Litster et al., 2007b).

The significant increase in the detection of MDR MRSP over time is a concerning finding since it creates major therapeutic limitations. The MRSP detected in this study belonged mainly to ST71-II-III, which is known to be one of the most disseminated clonal lineages in dogs and cats in Europe (Kadlec et al., 2010; Perreten et al., 2010). Although rarely, human infection by MRSP ST71-II-III has already been described, thus highlighting its zoonotic potential (Stegmann, Burnens, Maranta & Perreten, 2010).

As seen in reports on humans, *S. epidermidis* showed a high frequency of methicillin-resistance (Becker, Heilmann & Peters, 2014) and all belonged to STs also found in humans (Rolo, de Lencastre & Miragaia, 2012). The two MRSA isolated in this study belonged to *S. aureus* CC5, which is frequently associated with human hospital-acquired MRSA (Aires-de-Sousa, 2017). Furthermore, both MRSA STs have been reported in humans from Portugal (Conceição et al., 2010; Espadinha et al., 2013). Interestingly, in this study, the *mecA* gene was mainly detected in *Staphylococcus* spp. isolated from cats. As this was a retrospective study, it was not possible to obtain information on the possible source of these infections. Nevertheless, the detection of MRSA and MRSE in dogs and cats with UTI is a public health issue since companion animals will have a role in dissemination of these bacteria to the household and public environment.

Ampicillin-resistance and HLGR in *Enterococcus* spp. strongly limit the therapeutic options against enterococcal infections (Chow, 2000). Although in this study ampicillin-resistant and/or HLGR *Enterococcus* spp. were uncommon, some belonged to high-risk CCs associated with hospital-acquired infections in humans, such as *E. faecalis* CC6 (formerly CC2) and *E. faecium* CC17 (Table S7) (Leavis, Bonten & Willems, 2006; Kuch et al., 2012). HLGR was mostly detected in *E. faecalis* and was caused by the presence of a bifunctional enzyme that is known to also confer resistance to a wide range of aminoglycosides (Chow, 2000). *E. faecalis* ST6 (CC6) has been previously described in hospitalised patients from Portugal but also in samples from pigs and from hospital waste waters (Freitas, Novais, Ruiz-Garbajosa, Coque & Peixe,

2009). As in this study, the *E. faecalis* ST16 is a clonal lineage that has been found to frequently harbour the bifunctional enzyme (Ruiz-Garbajosa et al., 2006; Larsen et al., 2010). Also, *E. faecalis* ST16 has been previously detected in healthy and hospitalised humans as well as in animals (Ruiz-Garbajosa et al., 2006; Freitas et al., 2009; Larsen et al., 2010).

E. faecium isolates were less frequent than those of *E. faecalis*, but were more commonly ampicillin-resistant (Table S7). A previous study has pointed to healthy dogs as reservoirs of ampicillin-resistant *E. faecium* CC17 including ST19 (Damborg et al., 2009). Besides belonging to CC17, the *E. faecium* ST19 and ST440 strains isolated in this study are also noteworthy for being simultaneously ampicillin-resistant and HLGR.

Enterobacteriaceae and *Staphylococcus* spp. together caused around 79% of the UTIs in dogs and cats. Thus, the increase in fluoroquinolone-resistance in both groups of bacteria is of great relevance. Furthermore, *Enterococcus* spp. also showed high levels of resistance to this antimicrobial class. Bearing in mind fluoroquinolones are considered highest priority critically important antimicrobials for humans (WHO, 2017a), and of great importance in the treatment of pyelonephritis in companion animals (Weese et al., 2011), judicious use of these antimicrobials should be pursued.

The fact that this study relied on samples submitted to the laboratory based on clinical judgement could be considered a bias towards resistance because urine cultures from complicated UTIs may be requested more frequently than from simple uncomplicated UTIs (Hall et al., 2013). However, if present, this bias was constant throughout the study time frame and therefore the increase in antimicrobial resistance is unequivocal.

Only in recent years has the EMA started to publish data about antimicrobial sales for companion animals. Nevertheless, the high β-lactam and fluoroquinolone-resistance frequencies detected in this study could be expected since these are the first and second most sold antimicrobials for companion animals in Portugal (EMA, 2017b). The high frequency of tick-borne diseases in Portugal that require the use of doxycycline in companion animals could have contributed to the high tetracycline-resistance seen in this study. Also, the increase in MDR Enterobacteriaceae may, to some extent, explain the increasing resistance trends observed for all the tested antimicrobials.

The limited access to complete data about the epidemiological and clinical history of the dogs and cats infected with high-risk clonal lineages was also a limitation of this study. Nevertheless, since most of these animals were treated at home by their owners, the role of dogs and cats with UTI in the spread of resistant bacteria into the environment should be considered. Furthermore, it has been shown that companion animals may share uropathogenic bacteria with their household members (Johnson et al., 2009), and therefore the role of dogs and cats as reservoirs of high-risk clonal lineages is a concern to veterinary professionals and owners.

This study showed that bacteria causing UTI in dogs and cats, especially Enterobacteriaceae, are increasingly resistant to the antimicrobials most widely used in UTI treatment. These

bacteria were found to harbour clinically relevant antimicrobial resistance mechanisms and simultaneously belong to high-risk clonal lineages, namely 3GC-resistant *E. coli* O25b:H4-B2-ST131, CC23 and ST648, MRSA CC5, MRSE CC5, HLGR *E. faecalis* CC6, and ampicillin/HLGR *E. faecium* CC17. Therefore, when such resistance mechanisms are suspected based on susceptibility testing, veterinary professionals and owners should be advised to take measures in order to reduce the spread of these bacteria, such as strict hand washing, cleaning of the animals' living environment as well as adequate faecal disposal. Also, longitudinal studies on the faecal carriage of these resistant high-risk clonal lineages during and after UTI should be conducted to assess the time of carriage and evaluate the extent and duration of the infection control measures that should be taken.

Supplementary data

Supplementary Table 1 – Antimicrobial agents and antimicrobial categories considered for MDR isolates classification

Antimicrobial categories	Enterobacteriaceae[a]	*Staphylococcus* spp.
Penicillins + β-lactamase inhibitors	AMC	-
Anti-staphylococcal β-lactams (or cephamycis)	-	OXA or FOX[b]
Third-generation cephalosporins	CTX, CAZ and/or CVN	-
Folate pathway inhibitors	SXT	SXT
Fluoroquinolones	CIP and/or ENR	CIP and/or ENR
Aminoglycosides	GEN	GEN

Legend: AMC, amoxicillin/clavulanate; OXA, Oxacillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CVN, cefovecin; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; ENR, enrofloxacin; GEN, gentamicin.

[a]Data on intrinsic resistance was not considered for analysis (e.g. amoxicillin/clavulanate in *Enterobacter* spp.).

[b]Oxacillin or cefoxitin were used according to the *Staphylococcus* species following CLSI (2013a) guidelines. Tetracycline-resistance was not included in MDR analysis because it is not routinely used for UTI treatment and tetracycline-resistance may not indicate resistance to doxycycline (more used in dogs and cats).

Supplementary Table 2 - Frequency of bacterial species in dogs and cats

Organism	Overall		Cat		Dog		*P*
	n	**% (CI)**	***n***	**% (CI)**	***n***	**% (CI)**	
Escherichia coli	412	43.5 (40.3-46.6)	96	38.9 (32.8-45.0)	316	45.1 (41.4-48.8)	0.1006
Proteus spp.	155	16.4 (14.0-18.7)	18	7.3 (4.0-10.5)	137	19.5 (16.6-22.5)	**<0.0001**
P. mirabilis	153	16.1 (13.8-18.5)	17	6.9 (3.7-10.0)	136	19.4 (16.5-22.3)	**<0.0001**
P. vulgaris	2	0.2 (0-0.5)	1	0.4 (0-1.2)	1	0.1 (0-0.4)	0.4534
Staphylococcus spp.	125	13.2 (11.0-15.3)	34	13.8 (9.5-18.1)	91	13.0 (10.5-15.5)	0.7438
S. pseudintermedius	91	9.6 (7.7-11.5)	9	3.6 (1.3-6.0)	82	11.7 (9.3-14.1)	**<0.0001**
S. felis	5	0.5 (0.1-1.0)	5	2.0 (0.3-3.8)	0	0	**0.0012**
S. aureus	5	0.5 (0.1-1.0)	3	1.2 (0-2.6)	2	0.3 (0-0.7)	0.1144
S. epidermidis	6	0.6 (0.1-1.1)	5	2.0 (0.3-3.8)	1	0.1 (0-0.4)	**0.0055**
Other	18	1.9 (1.0-2.8)	12	4.9 (2.2-7.5)	6	0.9 (0.2-1.5)	-
Enterococcus spp.	66	7.0 (5.3-8.6)	34	13.8 (9.5-18.1)	32	4.6 (3.0-6.1)	**<0.0001**
E. faecalis	53	5.6 (4.1-7.0)	30	12.2 (8.1-16.2)	23	3.3 (2.0-4.6)	**<0.0001**
E. faecium	9	1.0 (0.3-1.6)	3	1.2 (0-2.6)	6	0.9 (0.2-1.5)	0.7034
Other	4	0.4 (0-0.8)	1	0.4 (0-1.2)	3	0.4 (0-0.9)	-
Pseudomonas spp.	48	5.1 (3.7-6.5)	15	6.1 (3.1-9.0)	33	4.7 (3.1-6.3)	0.4012
Klebsiella spp.	33	3.5 (2.3-4.6)	13	5.3 (2.5-8.0)	20	2.8 (1.6-4.1)	0.1037
Streptococcus spp.	34	3.6 (2.4-4.8)	6	2.4 (0.5-4.4)	28	4.0 (2.5-5.4)	0.3218
Corynebacterium spp.	21	2.2 (1.3-3.2)	13	5.3 (2.5-8.1)	8	1.1 (0.4-1.9)	**0.0004**
Enterobacter spp.	27	2.8 (1.8-3.9)	9	3.6 (1.3-6.0)	18	2.6 (1.4-3.7)	0.3784
Other	27	2.8 (1.8-3.9)	9	3.6 (1.3-6.0)	18	2.6 (1.4-3.7)	-

Legend: *n*, number of isolates; %, frequency of isolates; CI, 95% confidence interval; *P*, probability: bold highlights statistically significant differences as determined by Fisher's exact test.

Supplementary Table 3 – *E. coli* and Enterobacteriaceae resistance temporal trends.

Antimicrobial	*Escherichia coli*		Enterobacteriaceae	
	OR (CI)	*P*	OR (CI)	*P*
Amoxicillin/clavulanate	1.155 (1.090-1.225)	**<0.0001**	1.187 (1.129-1.248)	**<0.0001**
Trimethoprim/sulfamethoxazole	1.037 (0.990-1.086)	0.1267	1.052 (1.015- 1.090)	**0.0052**
Third-generation cephalosporins	1.188 (1.107-1.274)	**<0.0001**	1.202 (1.140-1.268)	**<0.0001**
Fluoroquinolones	1.053 (1.005-1.104)	**0.0301**	1.080 (1.041- 1.121)	**<0.0001**
Gentamicin	1.087 (1.015-1.165)	**0.0177**	1.120 (1.062- 1.182)	**<0.0001**
Tetracycline	1.075 (1.029-1.123)	**0.0011**	1.092 (1.049- 1.138)	**<0.0001**
Multidrug resistance	1.120 (1.056-1.188)	**0.0002**	1.162 (1.106- 1.221)	**<0.0001**

Legend: OR, Odds ratio; CI, 95% Confidence interval; *P*, probability: bold highlights statistically significant differences as determined by logistic regression.

Supplementary Table 4 – *Proteus* spp. and *Staphylococcus* spp. antimicrobial resistance

	AMC % (CI)	SXT % (CI)	3GC % (CI)	FQ % (CI)	GEN % (CI)	TET % (CI)	MET % (CI)	MDR % (CI)
Proteus spp.								
1999-2006	4.0 (0.2-7.9) *n*=99	28.3 (19.4-37.2) *n*=99	2.0 (0-4.8) *n*=98	17.2 (9.7-24.6) *n*=99	2.0 (0-4.8) *n*=98	-	-	3.1 (0-6.5) *n*=97
2007-2014	20.4 (9.6-31.1) *n*=54	43.6 (30.5-56.7) *n*=55	18.5 (8.2-28.9) *n*=54	30.9 (18.7-43.1) *n*=55	14.6 (5.2-23.9) *n*=55	-	-	21.2 (10.0-32.2) *n*=52
P	**0.0029**	0.0748	**0.0006**	0.0673	**0.0045**	-	-	**0.0006**
Staphylococcus spp.								
1999-2006	-	8.9 (2.6-15.1) *n*=79	-	6.3 (1.0-11.7) *n*=79	3.8 (0-8.0) *n*=79	35.4 (24.9- 46.0) *n*=79	6.4 (1.0-11.8) *n*=78	2.6 (0-6.1) *n*=78
2007-2014	-	13.0 (3.3-22.8) *n*=46	-	22.2 (10.1-34.4) *n*=45	10.9 (1.9-19.9) *n*=46	23.9 (11.6- 36.2) *n*=46	14.6 (3.8-25.4) *n*=41	10.0 (0.7-19.3) *n*=40
P	-	0.5471	-	**0.0189**	0.1427	0.2306	0.1847	0.1780

Legend: AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQ, fluoroquinolones; GEN, gentamicin; TET, tetracycline; MET, Methicillin; *n*, number of tested isolates; %, frequency of fully-resistant isolates; CI, 95% Confidence interval; *P*, probability, bold highlights statistically significant differences as determined by Fisher's exact test.

Supplementary Table 5 – Antimicrobial resistance phenotype of multidrug-resistant Enterobacteriaceae (1999-2014)

Resistant antimicrobials	Resistance phenotype	Susceptibility phenotype		*n*	% (CI)
3	AMC, 3GC, SXT	FQ, GEN		7	7.5 (2.2-12.9)
	AMC, 3GC, FQ	SXT, GEN		7	7.5 (2.2-12.9)
	AMC, 3GC, GEN	FQ, SXT		1	1.1 (0-3.2)
	AMC, SXT, FQ	3GC, GEN		5	5.4 (0.8-10.0)
	3GC, SXT, FQ	AMC, GEN[a]		7	7.5 (2.2-12.9)
	3GC, FQ, GEN	AMC, SXT[a]		2	2.2 (0-5.1)
	SXT, FQ, GEN	AMC, 3GC		18	19.4 (11.3-27.4)
			Total	**47**	**50.5 (40.4-60.7)**
4	AMC, 3GC, SXT, GEN	FQ		1	1.1 (0-3.2)
	AMC, 3GC, SXT, FQ	GEN		17	18.3 (10.4-26.1)
	AMC, 3GC, FQ, GEN	SXT		3	3.2 (0-6.8)
	AMC, SXT, FQ, GEN	3GC		6	6.4 (1.5-11.4)
	3GC, SXT, FQ, GEN	AMC[a]		2	2.2 (0-5.1)
			Total	**29**	**31.2 (21.8-40.6)**
5	AMC, 3GC, SXT, FQ, GEN	-		17	18.3 (10.4-26.1)
			Total	**17**	**18.3 (10.4-26.1)**

Legend: *Enterobacter* spp. antimicrobial resistance data was not included in this analysis due to its intrinsic resistance against amoxicillin/clavulanate. Intermediate susceptible isolates were considered as susceptible. AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQ, fluoroquinolones; GEN, gentamicin;
[a]All isolates were ESBL-producers, thus AMC might not be clinically effective.

Supplementary Table 6 – AmpC-producer *P. mirabilis*

Isolate	Year	β-Lactamase	Animal	MDR	AMC	3GC	SXT	FQ	GEN
FMV6933/04	2004	CMY-2	Cat	**Yes**	R	R	R	R	S
FMVBeiças/07	2007	CMY-2	Dog	**Yes**	R	R	R	R	S
FMVLord/07	2007	CMY-2	Dog	**Yes**	R	R	R	R	S
FMV1089/07	2007	CMY-2	Dog	**Yes**	R	R	R	R	R
FMV4138/10	2010	CMY-2	Dog	**Yes**	R	R	R	R	S
FMV74/2012	2012	CMY-2	Dog	**Yes**	R	R	R	R	R
FMV5017/13	2013	DHA-1	Dog	**Yes**	R	R	R	S	S
FMV62/2013	2013	CMY-2	Dog	**Yes**	R	R	R	R	R
FMV3252/14	2014	CMY-2	Dog	**Yes**	R	R	R	S	I

Legend: AMC, amoxicillin/clavulanate; 3GC, third-generation cephalosporins; SXT, trimethoprim/sulfamethoxazole; FQ, fluoroquinolones; GEN, gentamicin; R, resistant; I, intermediate; S, susceptible.

Supplementary Table 7 – Ampicillin and/or HLGR *Enterococcus* spp. clonal lineages

Isolate	Year	Species	Clonal lineage	Clonal complex (CC)[a]	HLGR enzyme	Animal	AMP	HLGEN	CIP	TET
FMV95/99	1999	*E. faecium*	ST18	CC17	-	Dog	R	S	R	S
FMV1678/01	2001	*E. faecium*	ST132	CC17	-	Cat	R	S	R	S
FMV337/03	2003	*E. faecium*	ST440	CC17	*aac(6')-Ieaph(2'')-Ia*	Dog	R	R	R	R
FMV3626/03	2003	*E. faecalis*	ST59	CC59	*aac(6')-Ieaph(2'')-Ia*	Cat	S	R	R	S
FMV6735/06	2006	*E. faecalis*	ST35	none	*aac(6')-Ieaph(2'')-Ia*	Cat	S	R	R	R
FMV6736B/06	2006	*E. faecalis*	ST36	CC355	*aac(6')-Ieaph(2'')-Ia*	Dog	S	R	I	R
FMV6210/08	2008	*E. faecium*	ST1282	CC17	-	Cat	R	S	R	S
FMV1899/09	2009	*E. faecium*	ST19	CC17	*aph(2'')-1d*	Dog	R	R	I	R
FMV2859/09	2009	*E. faecium*	ST1282	CC17	-	Dog	R	S	R	S
FMV4233/09	2009	*E. faecalis*	ST86	none	*aac(6')-Ieaph(2'')-Ia*	Cat	S	R	R	R
FMV5944/10	2010	*E. faecalis*	ST6	CC6	*aac(6')-Ieaph(2'')-Ia*	Dog	S	R	R	R
FMV40/2014	2014	*E. faecalis*	ST16	CC16	*aac(6')-Ieaph(2'')-Ia*	Cat	S	R	R	R
FMV43/2014	2014	*E. faecalis*	ST16	CC16	*aac(6')-Ieaph(2'')-Ia*	Cat	S	R	R	R
FMV81/2014	2014	*E. faecium*	ST1283	CC17	-	Dog	R	S	R	R

Legend: AMP, ampicillin; HLGR, high-level gentamicin-resistance; HLGEN, high-level gentamicin; CIP, ciprofloxacin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible;
[a]clonal complexes based on eBUSRT analysis of entire *E. faecium* or *E. faecalis* MLST database.

Supplementary Figure 1 – New ST of *E. coli* belonging to CC23

a)

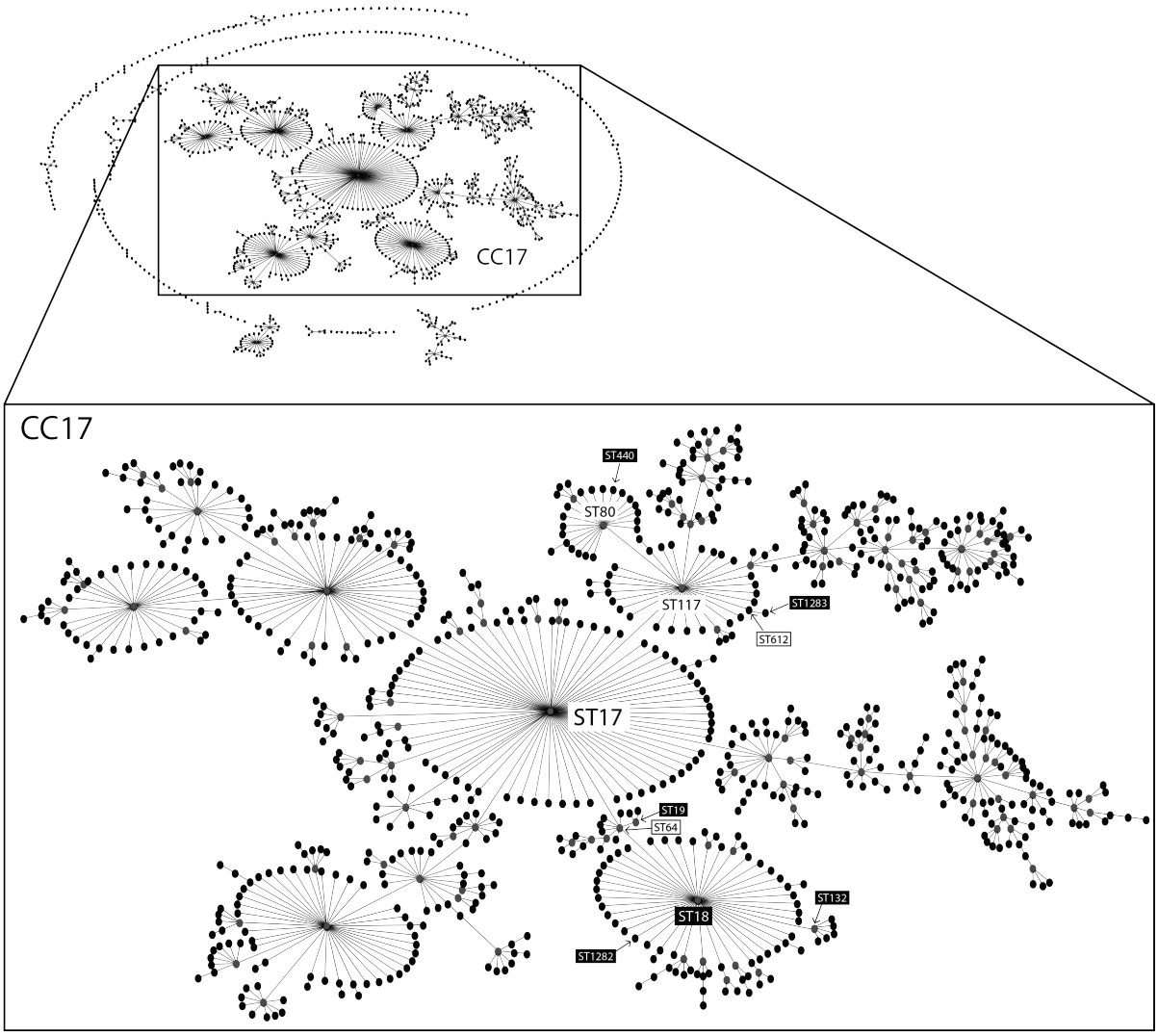
	adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	CC
ST23	6	4	12	1	20	13	7	23
New ST (FMV1630/07)	6	4	12	1	20	13	New	23

b)

```
Allele recA 7   CGCACGTAAACTGGGCGTCGATATCGATAACCTGCTGTGCTCCCAGCCGGACACCGGCGA
New recA allele CGCACGTAAACTGGGCGTCGATATCGATAACCTGCTGTGCTCCCAGCCGGACACCGGCGA
                ************************************************************
Allele recA 7   GCAGGCACTGGAAATCTGTGACGCCCTGGCGCGTTCTGGCGCAGTAGACGTTATCGTCGT
New recA allele GCAGGCACTGGAAATCTGTGACGCCCTGGCGCGTTCTGGCGCAGTAGACGTTATCGTCGT
                ************************************************************
Allele recA 7   TGACTCCGTGGCGGCACTGACGCCGAAAGCGGAAATCGAAGGCGAAATCGGCGACTCTCA
New recA allele TGACTCCGTGGCGGCACTGACGCCGAAAGCGGAAATCGAAGGCGAAATCGGCGACTCTCA
                ************************************************************
Allele recA 7   CATGGGCCTTGCGGCACGTATGATGAGCCAGGCGATGCGTAAGCTGGCGGGTAACCTGAA
New recA allele CATGGGCCTTGCGGCACGTATGATGAGCCAGGCGATGCGTAAGCTGGCGGGTAACCTGAA
                ************************************************************
Allele recA 7   GCAGTCCAACACGCTGCTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTT
New recA allele GCAGTCCAACACGCTGCTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTT
                ************************************************************
Allele recA 7   CGGTAACCCGGAAACCACTACCGGTGGTAACGCGCTGAAATTCTACGCCTCTGTTCGTCT
New recA allele CGGTAACCCGGAAACCACTACCGGTGGTAACGCGCTGAAATTCTACGCCTCTGTTCGTCT
                ************************************************************
Allele recA 7   CGACATCCGTCGTATCGGCGCGGTGAAAGAGGGCGAAAACGTGGTGGGTAGCGAAACCCG
New recA allele CGACATCCGTCGTATCGGCGCGGTGAAAGAGGGCGAAAACGTGGTGGGTAGCGAAACCCG
                ************************************************************
Allele recA 7   CGTGAAAGTGGTGAAGAACAAAATCGCTGCACCGTTTAAACAGGCTGAATTTCAGATCCT
New recA allele CGTGAAAGTGGTGAAGAACAAAATCGCTGCACCGTTTAAACAGGCTGAATTTCAGATCCT
                ************************************************************
Allele recA 7   CTACGGCGAAGGTATCAACTTCTACGGCGA
New recA allele CTACGGCGAAGGTATCAATTTCTACGGCGA
                ****************** ***********
```

Legend: a) Comparison of allelic MLST profile of new *E. coli* ST with *E. coli* ST23 (CC23). The new ST varies from ST23, the predicted founder of CC23, in a single locus variation (SLV) namely *rec*A. MLST genes: *adk*, adenylate kinase; *fum*C, fumarate hydratase; *gyr*B, DNA gyrase; *icd*, isocitrate/isopropylmalate dehydrogenase; *mdh*, malate dehydrogenase; *pur*A, adenylosuccinate dehydrogenase; *rec*A, ATP/GTP binding motif.
b) Sequence alignment of new *rec*A allele with its closest match, namely allele 7. The new allele differs from allele 7 in a single nucleotide change (marked in black).

Supplementary Figure 2 – eBURST analysis of *E. faecium* strains



Legend: eBURST clustering analysis of all known ST allelic profiles of *E. faecium* (*n*= 1284) from the *E. faecium* central MLST database (https://pubmlst.org/efaecium/) at 19th February 2017. Each ST is represented as a dot and each line indicates a single-locus difference between STs. The clonal complex (CC) assignment was based on the ST assigned as the founder genotype by eBURST analysis. Black boxes indicate the STs found in the present study, including the novel STs (ST1282 and ST1283).

Chapter 4

K. pneumoniae and *P. mirabilis* from UTI: population structure, antimicrobial resistance and virulence genes

4.1 *Klebsiella pneumoniae* causing urinary tract infections in companion animals and humans: population structure, antimicrobial resistance and virulence genes

Full paper published at *The Journal of Antimicrobial Chemotherapy*

Marques, C.*, Menezes, J., Belas, A., Aboim, C., Cavaco-Silva, P., Trigueiro, G., Gama, L.T., Pomba, C. (2018). *Klebsiella pneumoniae* causing urinary tract infections in companion animals and humans: population structure, antimicrobial resistance and virulence genes. *The Journal of Antimicrobial Chemotherapy*, 74, 594-602.

*The author performed the bacterial identification, DNA extraction and PCR assays of all *K. pnemoniae* isolates. The author conducted 90% of antimicrobial susceptibility testing. The author perfomed PFGE and MLST analysis. The author performed data and statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on co-authors and reviewers revisions.

Partial results were presented as,

Two Oral communications at the international congress 25th ECVIM-CA, 2015, Lisbon, Portugal; and at the international congress 27th ECVIM-CA, 2017, St. Julian's, Malta;

Two Poster communications at the international congress 26th ECCMID, 2016, Amsterdam, the Netherlands; and at the international congress ASM Microbe 2016, 2016, Boston USA;

One work deserved the award of "Second best oral presentation" at the 25th ECVIM-CA given by the European Society of Veterinary Nephrology and Urology (ESVNU).

Klebsiella pneumoniae causing urinary tract infections in companion animals and humans: population structure, antimicrobial resistance and virulence genes.

Cátia Marques[1], Juliana Menezes[1], Adriana Belas[1], Catarina Aboim[1], Patrícia Cavaco-Silva[2,3], Graça Trigueiro[4], Luís Telo Gama[1], Constança Pomba[1]

[1]CIISA - Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal;
[2]Centro de Investigação Interdisciplinar Egas Moniz, Instituto Universitário Egas Moniz, Caparica, Portugal;
[3]Technophage, Lisboa, Portugal
[4]Laboratório de Análises Clínicas Dr. Joaquim Chaves, Lisboa, Portugal

Abstract

Objectives: To characterise the population structure, antimicrobial resistance and virulence genes of *Klebsiella* spp. isolated from dogs, cats and humans with urinary tract infections (UTIs).

Methods: *Klebsiella* spp. from companion animals (*n* = 27) and humans (*n* = 77) with UTI were tested by the disc diffusion method against 29 antimicrobials. Resistant/intermediate isolates were tested by PCR for 16 resistance genes. Seven virulence genes were screened for by PCR. All *Klebsiella pneumoniae* from companion animals and third-generation cephalosporin (3GC)-resistant isolates from humans were typed by MLST. All *Klebsiella* spp. were compared after PFGE *Xba*I macro-restriction using Dice/UPGMA with 1.5% tolerance.

Results: $bla_{\text{CTX-M-15}}$ was detected in >80% of 3GC-resistant strains. *K. pneumoniae* high-risk clonal lineage ST15 predominated in companion animal isolates (60%, *n* = 15/25). Most companion animal ST15 *K. pneumoniae* belonged to two PFGE clusters (C4, C5) that also included human strains. Companion animal and human ST15-CTX-M-15 *K. pneumoniae* shared a *fimH-1/mrkD/entB/ycfM/kfu* virulence profile, with a few (*n* = 4) also harbouring the yersiniabactin siderophore-encoding genes. The hospital-adapted ST11 *K. pneumoniae* clonal lineage was detected in a cat (*n* = 1) and a human (*n* = 1); both were MDR, had 81.1% Dice/UPGMA similarity and shared several virulence and resistance genes. Two 3GC-resistant ST348 strains with 86.7% Dice/UPGMA similarity were isolated from a cat and a human.

Conclusions: Companion animals with UTI become infected with high-risk *K. pneumoniae* clonal lineages harbouring resistance and virulence genes similar to those detected in strains from humans. The ST15-CTX-M-15 *K. pneumoniae* clonal lineage was disseminated in companion animals with UTI. Caution must be applied by companion animal caretakers to avoid the spread of *K. pneumoniae* high-risk clonal lineages.

Introduction

Urinary tract infections (UTIs) are commonly diagnosed in companion animals and humans and frequently require antimicrobial treatment (Weese et al., 2011; Flores-Mireles et al., 2015). Although less frequently than *Escherichia coli*, UTIs in humans and companion animals can be caused by *Klebsiella pneumoniae* (Flores-Mireles et al., 2015; Marques et al., 2018b). Furthermore, *K. pneumoniae* is a major pathogen of nosocomial infections, including UTIs, that is frequently associated with resistance to the highest priority critically important antimicrobials (European Centre for Disease Prevention and Control [ECDC], 2016b).

Recently, the WHO published a global priority list of antimicrobial resistant bacteria, where third-generation cephalosporin (3GC)- and/or carbapenem-resistant Enterobacteriaceae, including *K. pneumoniae*, were included in the Priority 1 group (WHO, 2017b). Therefore, the detection of ESBL/AmpC-producing *K. pneumoniae* in veterinary medicine is an important issue since animals may contribute to its spread (Pomba et al., 2017). Moreover, ESBL-producing Enterobacteriaceae are frequently MDR, posing great therapeutic challenges (ECDC, 2017).

Some studies regarding ESBL-producing *K. pneumoniae* infections in companion animals have reported that these bacteria may belong to high-risk clonal lineages found in humans (Hidalgo et al., 2013; Donati et al., 2014; Ewers et al., 2014b; Wohlwend et al., 2015; Harada et al., 2016; Kuan et al., 2016; Maeyama et al., 2018; Zogg et al., 2018a). However, current knowledge of *K. pneumoniae* epidemiology in veterinary medicine is still limited when compared with human medicine.

K. pneumoniae may harbour several virulence genes that contribute to its pathogenicity. A recent study, using WGS, detected several virulence factors that were significantly more frequent among invasive *K. pneumoniae*, such as yersiniabactin, aerobactin and the regulators of mucoid phenotype (Holt et al., 2015). Additionally, some virulence factors are known to play an important role in UTI infection, such as type-1 fimbriae and aerobactin (Stahlhut et al., 2009; Flores-Mireles et al., 2015). However, studies reporting the frequency of such *K. pneumoniae* virulence genes are limited in isolates from companion animals and humans with UTI (Podschun et al., 1993; El Fertas-Aissani et al., 2013; Ranjbar et al., 2016; Wasfi, Elkhatib & Ashour, 2016).

This study aimed to characterise the antimicrobial resistance and virulence genes of *K. pneumoniae* strains causing UTI in companion animals (dogs and cats) and humans. It also aimed to characterise the population structure of *Klebsiella* spp. isolated from companion animals and humans with UTI.

Materials and methods

Bacteria isolates

Twenty-five *K. pneumoniae* and two *Klebsiella oxytoca* were isolated at the Laboratory of Antimicrobial Resistance in the Faculty of Veterinary Medicine, University of Lisbon, from 24 dogs and cats with UTI living in the Lisbon area. Seventy-six *K. pneumoniae* and one *K. oxytoca* were collected from human patients with UTI at a private clinical analysis laboratory and from hospital microbiology laboratories in the Lisbon area. Epidemiological data regarding the type of UTI were not available. The clinical *Klebsiella* spp. from companion animals and from the human private analysis laboratory were isolated sequentially during 2002–15 and 2014, respectively. Isolates from the hospital microbiology laboratories were part of a randomly non-sequential bacterial collection stored from 2006 to 2015. Samples from the human private

laboratory (*n* = 58) were obtained from humans with community-acquired UTI. The hospital laboratory samples (*n* = 19) were obtained from hospitalised patients with UTI; however, the nosocomial origin of infection was uncertain due to insufficient patient data.

Bacteriological methods

Standard quantitative urine culture was performed, and initial species identification was conducted by phenotypical tests (API20E or VITEK II, BioMérieux, Marcy-l'Étoile, France). Bacterial identification was confirmed by species-specific PCR (Kovtunovych et al., 2003; Padmavathy et al., 2012).

Susceptibility testing

Susceptibility testing was performed by the disc diffusion method according to CLSI guidelines with the following antimicrobials: (Oxoid, Hampshire, UK) ampicillin 10 µg, amoxicillin/clavulanate 30 µg, cephalothin 30 µg, cefoxitin 30 µg, cefotaxime 30 µg, ceftazidime 30 µg, cefovecin 30 µg, piperacillin/tazobactam 110 µg, cefepime 30 µg, imipenem 10 µg, meropenem 10 µg, aztreonam 30 µg, ertapenem 10 µg, nalidixic acid 30 µg, ciprofloxacin 5 µg, enrofloxacin 5 µg, norfloxacin 10 µg, levofloxacin 5 µg, gentamicin 10 µg, kanamycin 30 µg, netilmicin 30 µg, tobramycin 10 µg, amikacin 30 µg, chloramphenicol 30 µg, florfenicol 30 µg, tetracycline 30 µg, fosfomycin 50 µg, nitrofurantoin 300 µg and trimethoprim/sulfamethoxazole 25 µg (CLSI, 2013b).

Veterinary CLSI breakpoints (CLSI, 2013a; Clinical and Laboratory Standards Institute [CLSI], 2018) were used for cephalothin, cefovecin and enrofloxacin. Florfenicol (a fluorinated compound in use for bovine respiratory infections) breakpoints were derived from the veterinary CLSI breakpoints for swine infections (CLSI, 2013a). Fosfomycin results were interpreted according to the Societé Française de Microbiology (SFM, 2010). Human CLSI breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2017) were used for the remaining antimicrobials.

Antimicrobial categories were used to characterise multidrug resistance as previously proposed (Magiorakos et al., 2012). *Klebsiella* spp. were considered as MDR when fully-resistant to three or more antimicrobial categories.

Antimicrobial resistance determinants

β-Lactam-resistant/intermediate *Klebsiella* spp. (excluding only ampicillin-resistant isolates) were tested for the presence of bla_{TEM} and bla_{OXA} genes (Pomba et al., 2006). 3GC-resistant/intermediate *Klebsiella* spp. were further screened for ESBL ($bla_{CTX\text{-}M\text{-}type}$) and AmpC ($bla_{CIT\text{-}type}$, $bla_{DHA\text{-}type}$, $bla_{MOX\text{-}type}$, $bla_{ACT\text{-}type}$, $bla_{FOX\text{-}type}$ and $bla_{MIR\text{-}type}$) β-lactamase genes by PCR and sequencing (Pérez-Pérez & Hanson, 2002; Guessennd et al., 2008). 3GC-resistant/intermediate isolates lacking $bla_{CTX\text{-}M\text{-}type}$ or AmpC genes were characterised by

nucleotide sequencing for detection of the *bla*$_{TEM}$ and *bla*$_{SHV}$ ESBL genes. The presence of ISE*cp1* upstream of the *bla*$_{CTX\text{-}M\text{-}type}$ gene was investigated by PCR and sequencing (Edelstein, Pimkin, Palagin, Edelstein & Stratchounski, 2003; Gonullu et al., 2008).

The presence of genes conferring resistance to aminoglycosides [*aphAI-IAB*, *aac(6')-Ib*, *aac(3')-IV*] (Frana, Carlson & Griffith, 2001; Sáenz et al., 2004; Robicsek et al., 2006a), tetracyclines [*tet*(A), *tet*(B)] (Guardabassi, Dijkshoorn, Collard, Olsen & Dalsgaard, 2000), phenicols (*floR*) (Sáenz et al., 2004), fluoroquinolones (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*) (Robicsek et al., 2006b; Cavaco, Hasman, Xia & Aarestrup, 2009; Wang et al., 2009) and trimethoprim/sulfamethoxazole [*sul1*, *sul2*, *sul3*, *dfrA12*, *dfrIa* (targeting *dfrA1*, *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*)] (Guerra, Soto, Argüelles & Mendoza, 2001; Maynard et al., 2003; Navia, Ruiz, Sanchez-Cespedes & Vila, 2003; Perreten & Boerlin, 2003; Sáenz et al., 2004) was evaluated in all resistant/intermediate isolates.

Furthermore, all *Klebsiella* spp. were tested for the presence of *oqxAB* (Kim et al., 2009c) efflux pump genes and *ompK35* and *ompK36* outer membrane protein genes (Lee et al., 2007).

Virulence genes

Klebsiella spp. were screened by PCR for the following virulence genes: type-1 (*fimH-1*) adhesin; type-3 (*mrkD*) adhesin and FimH-like (*kpn*) adhesin (El Fertas-Aissani et al., 2013; Compain et al., 2014); outer membrane lipoprotein (*ycfM*) (El Fertas-Aissani et al., 2013); catecholate siderophore receptor (*iroN*) (Johnson et al., 2000); enterobactin (*entB*) (Compain et al., 2014); aerobactin (*iutA*) (Compain et al., 2014); iron transporter with phosphotransferase function (*kfu*) (Compain et al., 2014); yersiniabactin high-pathogenicity island (YHPI) (*fyuA*, *irp-1*, *irp-2*, *ybtS*) (Johnson & Stell, 2000; Schubert, Cuenca, Fischer & Heesemann, 2000; Compain et al., 2014) serum resistance-associated outer membrane lipoprotein (*traT*) (Johnson & Stell, 2000); regulator of mucoid phenotype A (*rmpA*) (Compain et al., 2014); cytotoxic necrotizing factor-1 (*cnf-1*) (Yamamoto et al., 1995); and allantoin metabolism associated gene (*allS*) (Compain et al., 2014).

Population structure analysis

Klebsiella spp. were characterised by PFGE after *Xba*I (New England Biolabs, Beverly, MA, USA) restriction on a CHEF DR II apparatus (Biorad, Hercules, CA, USA) as previously described (Rodrigues et al., 2016).

All *K. pneumoniae* from companion animals and 3GC-resistant *K. pneumoniae* from humans were typed by MLST (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Data analysis

Statistical analysis was conducted with the SAS statistical software package for Windows, version 9.3 (SAS Institute, Cary, NC, USA). Fisher's exact test was used with an α value of 0.05.

eBURSTv3 software was used to analyse the *K. pneumoniae* STs found in this study.

Interpretation of *Klebsiella* spp. PFGE patterns was conducted with Bionumerics software, version 6.6 (BioMérieux, Marcy-l'Étoile, France) using the Dice/UPGMA clustering method with a tolerance of 1.5% and a clustering cut-off of 80%.

Results

Klebsiella spp. antimicrobial resistance

K. pneumoniae from companion animals presented high antimicrobial resistance, especially against cephalothin, 3GC, fluoroquinolones, kanamycin, tetracycline and trimethoprim/sulfamethoxazole (>60%). *K. pneumoniae* from humans showed higher resistance frequencies against nitrofurantoin and trimethoprim/sulfamethoxazole. Fosfomycin, amikacin and carbapenems were the antimicrobials with the lowest resistance frequencies in all *K. pneumoniae* (Table 1).

The bla_{TEM} and bla_{OXA-1} β-lactamase genes were frequent among β-lactam-non-susceptible *K. pneumoniae* (strains categorised as antimicrobial resistant or intermediate) (Table 2) and 41.3% (n = 19/46) harboured both. The ESBL $bla_{CTX-M-15}$ gene was detected in >80% of 3GC-resistant strains (Table 2). Two *K. pneumoniae* from companion animals harboured $bla_{CTX-M-15}$ and an AmpC gene simultaneously ($bla_{CTX-M-15}$/bla_{DHA-1}, $bla_{CTX-M-15}$/bla_{CMY-2}). The genotype bla_{TEM}/bla_{OXA-1}/$bla_{CTX-M-15}$ was the most common (56.2%, n = 18/32) among all 3GC-resistant strains. ISE*cp1* was located upstream of all $bla_{CTX-M-15}$ genes except in two strains.

Among the tested *qnr* genes, *qnrB* was the most common and was significantly more frequent among fluoroquinolone-non-susceptible *K. pneumoniae* from humans (P = 0.0284) (Table 2). Most *qnrB*-positive *K. pneumoniae* (86.7%, n = 13/15) also harboured $bla_{CTX-M-15}$.

Kanamycin was the aminoglycoside with the highest resistance frequency, followed by tobramycin and gentamicin (Table 1). Aminoglycoside-non-susceptible *K. pneumoniae* from companion animals and humans frequently harboured *aac(6')-Ib* (80.0%) and fewer were positive for *aphAi-iAB* (25.7%) (Table 2). All strains were negative for *aac(3')-IV*.

Trimethoprim/sulfamethoxazole-non-susceptible *K. pneumoniae* frequently harboured *sul1*, *sul2* and/or *dfrIa* (Table 2). The genotype *sul1*/*sul2*/*dfrIa* was the most common in companion animals (37.5%, n = 6/16) followed by *sul1*/*sul2*/*dfrA12* (18.8%, n = 3/16) and *sul2* alone (18.8%, n = 3/16). In *K. pneumoniae* from humans, *sul2* was frequently detected alone (40.9%, n = 9/22) and in the *sul1*/*sul2*/*dfrIa* genotype (22.7%, n = 5/22).

Table 1 - Antimicrobial resistance of *K. pneumoniae* strains

Antimicrobials	Companion animal (*N* = 25)[a]		Human-HA (*N* = 19)[a]		Human-CA (*N* = 57)[a]	
	%R (*n*)	%I (*n*)	%R (*n*)	%I (*n*)	%R (*n*)	%I (*n*)
Ampicillin	100 (25)	0 (0)	100 (19)	0 (0)	96.5 (55)	0 (0)
Amoxicillin/clavulanate	32.0 (8)	32.0 (8)	15.8 (3)	21.0 (4)	15.8 (9)	14.0 (8)
Cephalothin	64.0 (16)	4.0 (1)	36.8 (7)	0 (0)	22.8 (13)	5.3 (3)
Cefoxitin	20.0 (5)	0 (0)	5.3 (1)	0 (0)	1.8 (1)	1.8 (1)
Cefotaxime	60.0 (15)	0 (0)	21.0 (4)	0 (0)	22.8 (13)	0 (0)
Ceftazidime	44.0 (11)	12.0 (3)	21.0 (4)	0 (0)	8.8 (5)	8.8 (5)
Cefovecin	60.0 (15)	0 (0)	21.0 (4)	0 (0)	22.8 (13)	0 (0)
Piperacillin/tazobactam	12.0 (3)	16.0 (4)	21.0 (4)	15.8 (3)	1.8 (1)	10.5 (6)
Cefepime	32.0 (8)	16.0 (4)	15.8 (3)	0 (0)	14.0 (8)	5.3 (3)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Meropenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aztreonam	52.0 (13)	4.0 (1)	15.8 (3)	0 (0)	15.8 (9)	5.3 (3)
Ertapenem	0 (0)	4.0 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Nalidixic acid	80.0 (20)	4.0 (1)	21.0 (4)	10.5 (2)	21.0 (12)	12.3 (7)
Ciprofloxacin	72.0 (18)	8.0 (2)	21.0 (4)	15.8 (3)	14.0 (8)	14.0 (8)
Enrofloxacin	72.0 (18)	12.0 (3)	21.0 (4)	15.8 (3)	15.8 (9)	12.3 (7)
Norfloxacin	72.0 (18)	0 (0)	21.0 (4)	0 (0)	12.3 (7)	10.5 (6)
Levofloxacin	72.0 (18)	0 (0)	21.0 (4)	0 (0)	7.0 (4)	5.3 (3)
Gentamicin	32.0 (8)	0 (0)	21.0 (4)	5.3 (1)	14.0 (8)	0 (0)
Kanamycin	60.0 (15)	4.0 (1)	21.0 (4)	10.5 (2)	17.5 (10)	0 (0)
Netilmicin	24.0 (6)	0 (0)	5.3 (1)	0 (0)	1.8 (1)	0 (0)
Tobramycin	48.0 (12)	4.0 (1)	26.3 (5)	5.3 (1)	19.3 (11)	0 (0)
Amikacin	4.0 (1)	4.0 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Chloramphenicol	44.0 (11)	0 (0)	31.6 (6)	0 (0)	15.8 (9)	5.3 (3)
Florfenicol	24.0 (6)	44.0 (11)	5.3 (1)	10.5 (2)	10.5 (6)	22.8 (13)
Tetracycline	64.0 (16)	4.0 (1)	31.6 (6)	0 (0)	19.3 (11)	0 (0)
Fosfomycin[b]	0 (0)	0 (0)	0 (0)	0 (0)	8.8 (5)	0 (0)
Nitrofurantoin	44.0 (11)	24.0 (6)	57.9 (11)	21.0 (4)	31.6 (18)	15.8 (9)
Trimethoprim/sulfamethoxazole	64.0 (16)	0 (0)	47.4 (9)	0 (0)	22.8 (13)	0 (0)

Legend: %R, percentage of resistant isolates; %I, percentage of intermediate isolates; Human-HA, human hospital patients with UTI; Human-CA, human community-acquired UTI.

[a]The number shown is the total number of isolates tested.

[b]The use of the disc diffusion method for fosfomycin antimicrobial susceptibility testing is debatable (Kaase, Szabados, Anders & Gatermann, 2014); nevertheless, all fosfomycin-resistant isolates had confluent growth up to the antimicrobial disc.

Most tetracycline-non-susceptible *K. pneumoniae* from companion animals harboured *tet*(A) or *tet*(B) alone (88.2%, *n* = 15/17). Only *tet*(A) was detected in tetracycline-non-susceptible *K. pneumoniae* from humans; nevertheless, the majority were negative for both genes (50.0%, *n* = 9/18) (Table 2).

Table 2- Antimicrobial resistance genes among antimicrobial non-susceptible *K. pneumoniae* strains

Antimicrobial	Target gene	Overall	Companion animal	Human-HA	Human-CA	*P* value[a]
β-Lactams	*bla*$_{TEM}$	56.5% (26/46)	66.7% (12/18)	5/8	45.0% (9/20)	0.3640
	bla$_{OXA-1}$	63.0% (29/46)	66.7% (12/18)	4/8	65.0% (13/20)	0.7613
Third-generation cephalosporins	*bla*$_{CTX-M-15}$	81.2% (26/32)	80.0% (12/15)	3/4	84.6% (11/13)	1
	bla$_{CMY-2}$	15.6% (5/32)	26.7% (4/15)	0/4	7.69% (1/13)	0.1609
	bla$_{DHA-1}$	6.2% (2/32)	6.7% (1/15)	1/4	0% (0/13)	1
Fluoroquinolones	*qnrB*	31.9% (15/47)	14.3% (3/21)	3/7	47.4% (9/19)	0.0284
	qnrS	2.1% (1/47)	0% (0/21)	1/7	0% (0/19)	1
Aminoglycosides	*aac(6')-Ib*	80.0% (28/35)	76.5% (13/17)	4/7	100% (11/11)	0.6906
	aphAi-iAB	25.7% (9/35)	29.4% (5/17)	4/7	0% (0/11)	0.7112
Folate pathway inhibitors	*sul1*	65.8% (25/38)	75.0% (12/16)	6/9	53.9% (7/13)	0.4898
	sul2	76.3% (29/38)	81.2% (13/16)	6/9	76.9% (10/13)	0.7060
	sul3	2.6% (1/38)	6.2% (1/16)	0/9	0% (0/13)	0.4211
	dfrA12	15.8% (6/38)	25.0% (4/16)	2/9	0% (0/13)	0.2170
	dfrIa	38.8% (14/38)	37.5% (6/16)	3/9	38.5% (5/13)	1
Tetracycline	*tet*(A)	50.0% (17/34)	52.9% (9/17)	5/6	27.3% (3/11)	1
	tet(B)	17.6% (6/34)	35.3% (6/17)	0/6	0% (0/11)	0.0184
Phenicols	*floR*	2.1% (1/47)	5.6% (1/18)	0/7	0% (0/22)	0.4500

Legend: Human-HA, human hospital patients with UTI; Human-CA, human community-acquired UTI.
[a]*K. pneumoniae* from companion animals were compared with *K. pneumoniae* from humans (Human-CA + Human-HA) by Fisher's exact test.

K. pneumoniae chloramphenicol-resistance was high (Table 1); however, only one *K. pneumoniae* of companion animal origin harboured the *floR* gene (Table 2 and Table S1).
All *K. pneumoniae* were positive for *ompK35* and *ompK3*6 genes, except one that was negative for *ompK35* (FMV4919/09). The oqxAB efflux pump was also common (95.0%, *n* = 96/101) and likely chromosome encoded (Holt et al., 2015).
Eighty percent of *K. pneumoniae* from companion animals were MDR and most strains were resistant to more than five antimicrobial categories (Table S2). The frequency of MDR *K. pneumoniae* from humans was also high (30%, *n* = 23/76) (Table S2). Notably, all ESBL/AmpC-producers were MDR, except for one strain from human infection (Table S1).

K. oxytoca from companion animals were both MDR (resistant to 9 out of 12 tested antimicrobial categories) and harboured several resistance genes, including bla_{DHA-1} (Table S3).

K. pneumoniae virulence

All *K. pneumoniae* were positive for *fimH-1*, *mrkD* and *entB*. Furthermore, *ycfM*, *kpn* and *kfu* were also frequent (Table S4). Two main virulence genotypes (VGs) were detected, namely VG1 [*fimH-1*/*mrkD*/*ent(B)*/*ycfM*/*kfu*; 52.5%, *n* = 53/101] and VG2 [*fimH-1*/*mrkD*/*ent(B)*/*ycfM*/*kpn*; 40.6%, *n* = 41/101] (Table 3). *K. pneumoniae* from companion animals had a significantly higher frequency of VG1 (*P* = 0.0023) and a lower frequency of VG2 (*P* = 0.0045) than isolates from humans. In *K. pneumoniae* from humans, the frequencies of VG1 (43.4%, *n* = 33/76) and VG2 (48.7%, *n* = 37/76) were similar (Table 3). Although YHPI gene frequency was higher in *K. pneumoniae* from humans (*P* = 0.0165), it was also detected in strains from companion animals (16%) (Table 3 and Table S4). The remaining virulence genes were absent or seldom detected (Table S4).

Table 3- Virulence genotype of *K. pneumoniae* strains

Virulence genotype	Overall (*N* = 101)[a]	Companion animal (*N* = 25)[a]	Human-HA (*N* = 19)[a]	Human-CA (*N* = 57)[a]
fimH-1, entB, mrkD, ycfM, kfu	28.7% (*n* = 29)	60.0% (*n* = 15)	21.0% (*n* = 4)	17.5% (*n* = 10)
fimH-1, entB, mrkD, ycfM, kpn	24.8% (*n* = 25)	12.0% (*n* = 3)	31.6% (*n* = 6)	28.1% (*n* = 16)
fimH-1, entB, mrkD, ycfM, kfu, YHPI	21.8% (*n* = 22)	12.0% (*n* = 3)	31.6% (*n* = 6)	22.8% (*n* = 13)
fimH-1, entB, mrkD, ycfM, kpn, YHPI	12.9% (*n* = 13)	0% (*n* = 0)	10.5% (*n* = 2)	19.3% (*n* = 11)
fimH-1, entB, mrkD, ycfM, kpn, iutA, traT, YHPI	1.0% (*n* = 1)	4.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)
fimH-1, entB, mrkD, ycfM, kpn, traT	1.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	1.8% (*n* = 1)
fimH-1, entB, mrkD, ycfM, kpn, allS	1.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	1.8% (*n* = 1)
fimH-1, entB, mrkD, ycfM, kpn, kfu	1.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	1.8% (*n* = 1)
fimH-1, entB, mrkD, ycfM, kfu, iutA	1.0% (*n* = 1)	4.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)
fimH-1, entB, mrkD, ycfM, kfu, traT	1.0% (*n* = 1)	4.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)
fimH-1, entB, mrkD, ycfM, YHPI	1.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	1.8% (*n* = 1)
fimH-1, entB, mrkD, ycfM, allS	1.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	1.8% (*n* = 1)
fimH-1, entB, mrkD, ycfM	3.0% (*n* = 3)	0% (*n* = 0)	5.3% (*n* = 1)	3.5% (*n* = 2)
fimH-1, entB, mrkD, allS	1.0% (*n* = 1)	4.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)

Legend: Human-HA, human hospital patients with UTI; Human-CA, human community-acquired UTI; *fimH-1*, type-1 adhesin; *mrkD*, type-3 adhesin; *kpn*, FimH-like adhesin; *ycfM*, outer membrane lipoprotein; *entB*, enterobactin; *iutA*, aerobactin; *kfu*, iron transporter with phosphotransferase function; YHPI, yersiniabactin high-pathogenicity island; *traT*, serum resistance-associated outer membrane lipoprotein; *allS*, allantoin metabolism-associated gene.
[a]The number shown is the total number of isolates tested.

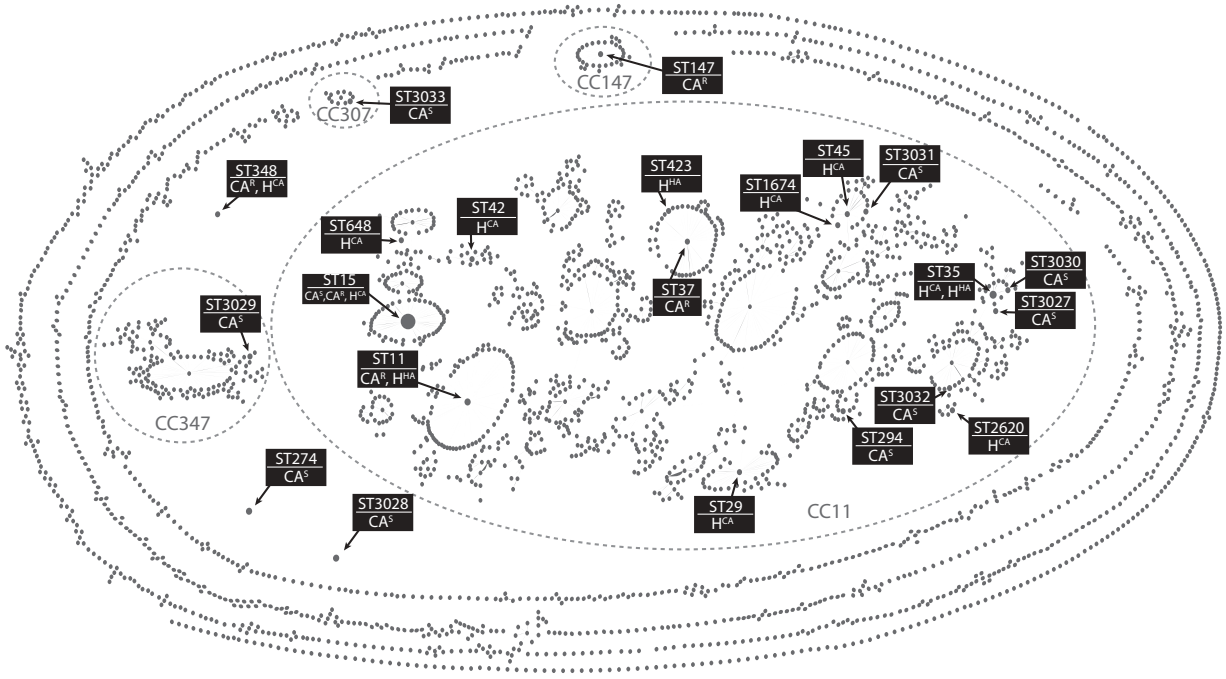
Population structure

Companion animal MDR 3GC-resistant *K. pneumoniae* belonged mainly to ST15 (73.3%, *n* = 11/15), but ST37, ST11, ST147 and ST348 were also detected (Figure 1 and Table S1). The 3GC-resistant *K. pneumoniae* from humans belonged to a large number of clonal lineages (*n* = 14), but also included MDR ST15 (*n* = 2), ST348 (*n* = 1) and ST11 (*n* = 1) (Figure 1 and Table S1). The 3GC-susceptible *K. pneumoniae* from companion animals showed additional lineage diversity; however, two ST15 were also found (Figure 1 and Table S1). All ST15 *K. pneumoniae* from animal or human origin were fluoroquinolone-resistant regardless of 3GC-susceptibility (Table S1 and Figure S1).

Seven new STs were described (ST3027–ST3033) (Figure 1 and Table S1). In eBURST analysis two of the new STs from companion animals (ST3027 and ST3030) were single- or double-locus variants of ST35, which was detected in three human UTI strains (Figure 1).

The PFGE analysis resulted in 19 clusters (C1–C19) and 43 single pulse-types (S1–S43) (Figure S1). Four clusters included *K. pneumoniae* strains from community and hospital patients with UTI. Moreover, six clusters included strains from companion animals and humans (C1, C2, C4, C5, C16, C18) that contained at least one human and companion animal strain sharing 80%–86.7% similarity (Figure S1).

Figure 1 - Population snapshot by eBURST analysis of all *K. pneumoniae* strains typed in this study against all *K. pneumoniae* STs known as of 24 November 2017



Legend: Detected clonal complexes (CCs) were assigned based on the predicted founder ST and are grouped with a dashed line. Each ST is represented by a circle and lines connect single-locus variants. The relative size of the circles indicates the prevalence of each ST. The black boxes indicate the *K. pneumoniae* STs found in this study. CA^S, 3GC-susceptible *K. pneumoniae* from companion animals with UTI; CA^R, 3GC-resistant *K. pneumoniae* from companion animals with UTI; H^{CA}, 3GC-resistant *K. pneumoniae* from human community-acquired UTI; H^{HA}, 3GC-resistant *K. pneumoniae* from human hospital patients with UTI. ST3027–ST3033 were the new STs described in this study.

The *K. pneumoniae* ST15 strains from companion animals belonged mainly to two clusters (C4 and C5) that also included strains from humans (Figure S1). All C4 and C5 strains shared VG1 (Figure S1). Four ST15 *K. pneumoniae* were obtained from three urine samples collected from the same animal at 2 month intervals (FMV3133/08, FMV4290/08, FMV5704/08A and FMV5704/08B). Although with some genetic variability, all strains clustered together with >85% similarity (Figure S1). In one human urine sample, two 100% similar strains with distinct antimicrobial resistance to 3GC were isolated (GTAFD58A, GTAFD58B) (Figure S1). Strains FMV3133/08 and FMV4290/08 were 100% similar to other strains (FMV338/08 and FMV4300/08, respectively) that were collected from unrelated companion animals in the same year.

The 3GC-resistant ST11 strains from one cat and one unrelated human shared 81.1% similarity, had similar MDR resistance and virulence profiles and were both isolated in 2007 (Table S1 and Figure S1).

The ST348 strains from a human and a cat were 86.7% similar and shared several virulence genes; however, the antimicrobial resistance profile differed extensively (Table S1 and Figure S1).

PFGE analysis of *K. oxytoca* revealed that both companion animal MDR strains clustered together but were unrelated to the human strain (Figure S2).

Discussion

The number of MDR 3GC-resistant *K. pneumoniae* was high among companion animal strains, hence creating great therapeutic limitations. The frequent detection of $bla_{\text{CTX-M-15}}$ among 3GC-resistant strains of animal and human origin is in agreement with previous published data (Ewers et al., 2014b; Rodrigues et al., 2014a). Interestingly, the $bla_{\text{CTX-M-15}}/bla_{\text{OXA-1}}/bla_{\text{TEM}}$ genotype has been reported in *K. pneumoniae* strains from humans in Portugal (Machado et al., 2006).

The 3GC-resistant and -susceptible *K. pneumoniae* isolated from companion animals in this study belonged mainly to ST15. This result agrees with previous studies showing that *K. pneumoniae* ST15-CTX-M-15-producers are widely disseminated among companion animals with several infections (Ewers et al., 2014b; Harada et al., 2016). *K. pneumoniae* ST15 has also been described in community- and hospital-onset infections in humans from Portugal, including UTIs (Rodrigues et al., 2014a; Vubil et al., 2017).

K. pneumoniae ST11 is a worldwide-disseminated high-risk clonal lineage that is associated with human hospital-acquired infections and with the dissemination of carbapenemases (Rodrigues et al., 2016). ST11 has been previously detected in hospital patients in Portugal (Managueiro et al., 2015), as in this study. The detection of ST11 in companion animals is therefore worrisome and has also been reported in Germany, Spain, Italy, Switzerland, Taiwan and Japan (Hidalgo et al., 2013; Donati et al., 2014; Wohlwend et al., 2015; Kuan et al., 2016;

Ovejero et al., 2017; Maeyama et al., 2018; Pulss et al., 2018; Zogg et al., 2018a). Unfortunately, it was not possible to investigate possible epidemiological links between the infected cat and human hospital settings. Remarkably, carbapenemase-producing *K. pneumoniae* ST11 and ST15 have been previously reported in companion animals (Pulss et al., 2018; Stolle et al., 2013).

The *K. pneumoniae* ST147 high-risk clonal lineage also seems to be disseminated worldwide in companion animals since it has been reported in China, Spain, Switzerland, Japan and now in Portugal (Harada et al., 2016; Ovejero et al., 2017; Xia et al., 2017; Maeyama et al., 2018; Zogg et al., 2018a). It should be noted that ST147 is one of the predominant KPC-3-producing *K. pneumoniae* clonal lineages in hospital-acquired infections in human patients in Portugal (Rodrigues et al., 2016).

K. pneumoniae ST348 is starting to be reported among ESBL- and carbapenemase-producing *K. pneumoniae* (Baraniak et al., 2013; Mshana et al., 2013; Rodrigues et al., 2016; Vubil et al., 2017), including in hospital-acquired infections in Portugal (Rodrigues et al., 2016; Vubil et al., 2017). To our knowledge, this is the first report of *K. pneumoniae* ST348 in companion animals and of MDR CMY-2-producing *K. pneumoniae* ST37 in Portugal. Interestingly, tigecycline-resistant *K. pneumoniae* ST37 has been detected in companion animals from Asia (Xia et al., 2017).

While 3GC-resistant *K. pneumoniae* from companion animals showed a main clonal lineage (ST15), human isolates were more diversified. The same was true in the overall PFGE analysis, pointing to different clonal epidemiology. The fact that the *K. pneumoniae* strains from humans and animals relate to different time periods is a limitation of the present study that could have hampered the detection of additional similarities. Nonetheless, the detection of *K. pneumoniae* ST15 in samples from unrelated companion animals over such a wide time-span suggests its dissemination.

Although PFGE is a more discriminatory typing method than MLST, it still lacks enough resolution to establish a definite link between strains of human and animal origin. Nevertheless, the detection of some PFGE clusters composed of *K. pneumoniae* strains from companion animals and humans belonging to high-risk clonal lineages is a concerning finding. WGS studies are warranted in the future to clarify the true role of companion animals as reservoirs of *K. pneumoniae* to humans in direct contact and vice versa.

The analysis of several strains from the same animal (three urine collections over a 4 month period), revealed the persistence of MDR *K. pneumoniae* ST15 strains. These results highlight the importance of conducting antimicrobial susceptibility testing and treating all UTI-predisposing co-morbidities to promote a definite cure and thus reduce the spread of such high-risk clonal lineages.

An association between ST15 *K. pneumoniae* and resistance to all fluoroquinolones was observed in this study, including resistance to levofloxacin, which is a third-generation

fluoroquinolone. The fluoroquinolone-resistance in *K. pneumoniae* was likely due to chromosomal mutations; nevertheless, *qnrB* was detected in human and companion animal *K. pneumoniae*. The link between $bla_{CTX\text{-}M\text{-}15}$ ESBL and *qnrB* genes observed in this study has been reported previously (Garcia-Fulgueiras et al., 2017). Several fluoroquinolone-resistant ST15 *K. pneumoniae* were also positive for *aac(6')-Ib*. Although this gene was not sequenced in this study, *K. pneumoniae* has been shown to frequently harbour the *aac(6')-Ib-cr* variant (Ewers et al., 2014b) and thus this resistance mechanism could also be involved.

Trimethoprim/sulfamethoxazole-resistant *K. pneumoniae* frequently harboured *sul1* and/or *sul2*. The same has been reported regarding *K. pneumoniae* from humans and companion animals (Ewers et al., 2014b; Holt et al., 2015).

The overall high frequency of *tet*(A) in *K. pneumoniae* is in line with previous findings showing that *tet*(A) is the most disseminated *tet* gene, followed by *tet*(D) (Holt et al., 2015).

Fosfomycin and amikacin are antimicrobials for human use that are suitable for UTI treatment (Gupta et al., 2010). The low amikacin-resistance frequency in this study is in agreement with previous reports of humans with *K. pneumoniae* UTI from Aveiro, Portugal (Linhares, Raposo, Rodrigues & Almeida, 2013). The low *K. pneumoniae* resistance to amikacin and fosfomycin makes its rational use critical, since these antimicrobials may gain importance in the future to avoid the use of carbapenems in the treatment of emerging MDR *K. pneumoniae* UTIs in humans.

Two main virulence genotypes were detected (VG1, VG2) that varied in the composition of less common virulence genes. Although VG1 and VG2 frequencies varied significantly between *K. pneumoniae* from companion animals and humans, VG1 and VG2 were detected in both. *fimH-1* and *mrkD* are thought to be ubiquitous among *K. pneumoniae* (El Fertas-Aissani et al., 2013; Holt et al., 2015) and in fact were detected in all *K. pneumoniae* in this study. Moreover, the type-1 adhesin is an important virulence factor for UTI and the type-3 adhesin strongly promotes biofilm formation (Stahlhut et al., 2009; Schroll et al., 2010). Although to a lesser extent than in *E. coli*, *K. pneumoniae fimH-1* seems to undergo pathoadaptive mutations (Stahlhut et al., 2009). Hence, in the future, *fimH-1* sequencing of isolates from companion animals and humans could help further understanding of the potential zoonotic nature of *K. pneumoniae* in UTI.

Frequencies of genes encoding yersiniabactin siderophore have been shown to be higher among invasive *K. pneumoniae* and a strong predictor of human infection (Holt et al., 2015). Additionally, yersiniabactin has been considered an important virulence factor in cystitis caused by *E. coli* (Brumbaugh et al., 2015). In this study, despite being more frequent among strains isolated from humans, the yersiniabactin-encoding genes were also detected in *K. pneumoniae* from companion animals, including the high-risk clonal lineages ST11 and ST15.

In agreement with previous studies, the virulence genes not belonging to VG1/VG2 were less common (Podschun et al., 1993; Schubert et al., 2000; El Fertas-Aissani et al., 2013). Nevertheless, some important virulence genes (Compain et al., 2014; Holt et al., 2015), such as *allS* and *iutA*, were detected in *K. pneumoniae* isolated from companion animals from this study.

The two *K. oxytoca* isolated from companion animals showed a significant MDR phenotype and harboured several major antimicrobial resistance genes. Therefore, despite being considered as an opportunistic bacterium, *K. oxytoca* may create significant therapeutic challenges and act as reservoirs of major antimicrobial resistance genes.

To the best of our knowledge, this is the first report of the *K. pneumoniae* clonal lineages affecting companion animals from Portugal. Notably, most of the 3GC-resistant *K. pneumoniae* clonal lineages isolated from companion animals with UTI overlapped the most frequent high-risk clonal lineages previously detected from human infection in Portugal and worldwide (Rodrigues et al., 2014a; Rodrigues et al., 2016; Vubil et al., 2016; Navon-Venezia et al., 2017). Although further studies are needed to explore the true zoonotic potential of *K. pneumoniae* isolated from companion animals, these results point to the need for a One Health approach to control the dissemination of important MDR *K. pneumoniae* high-risk clonal lineages.

K. pneumoniae from companion animals were frequently MDR and harboured clinically relevant antimicrobial resistance genes, thus highlighting the role of companion animals as reservoirs of major resistance determinants. This raises great concern in veterinary medicine since previous studies have shown that *K. pneumoniae* is prone to nosocomial dissemination (Ewers et al., 2014b). Precaution is advised when dealing with companion animals with UTI caused by MDR *K. pneumoniae* to avoid the dissemination of these high-risk clonal lineages.

Supplementary data

Supplementary Table 1 - Characterisation of *K. pneumoniae* strains typed by MLST

Strain	Year	Species	Resistance phenotype[a]	Resistance genotype	Virulence genotype	ST	PFGE Cluster
3598/07	2007	Cat	AMC, tzp, CEF, FOX, CTX, CAZ, CVN, atm, etp, NAL, ENR, CIP, LVX, NOR, KAN, tob, CHL, FFC, NIT, TET, SXT	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{DHA-1}$, *qnrB*, *aphAI-IAB*, *aac(6')-Ib*, *tet(A)*, *sul1*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kpn*, *iutA*, *traT*, *YHPI*	ST11	C1
PC263/07B	2007	Human-HA	AMC, tzp, CEF, FOX, CTX, CAZ, CVN, NAL, ENR, CIP, LVX, NOR, KAN, TOB, CHL, FFC, NIT, TET, SXT	*bla*$_{TEM}$, *bla*$_{DHA-1}$, *aphAI-IAB*, *aac(6')-Ib*, *tet(A)*, *sul1*, *dfrA12*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kpn*, *YHPI*	ST11	C1
3760/02	2002	Cat	NAL, ENR, CIP, LVX, NOR, KAN, CHL, ffc, NIT, SXT	*aphAI-IAB*, *sul1*, *sul2*, *dfrA12*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C4
5107/06	2006	Dog	NAL, ENR, CIP, LVX, NOR, KAN, GEN, NIT, TET, SXT	*aphAI-IAB*, *tet(A)*, *tet(B)*, *sul1*, *sul2*, *dfrA12*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*, *traT*	ST15	C4
6045/08	2008	Dog	CEF, CTX, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, CHL, FFC, nit, TET, SXT	*bla*$_{CTX-M-15}$, *tet(A)*, *sul1*, *sul 2*, *dfrA12*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C4
460/14	2014	Cat	amc, CEF, CTX, caz, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, KAN, TOB, ffc, NIT, TET	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib*, *tet(A)*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C4
53/2014	2014	Dog	amc, CEF, CTX, caz, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, KAN, TOB, ffc, TET, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib*, *tet(A)*, *sul1*, *dfrA12*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C4
338/08	2008	Cat	amc, tzp, CEF, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, AMK, GEN, KAN, NET, TOB, CHL, ffc, nit, TET, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib*, *tet(B)*, *sul1*, *sul2*, *dfrIa*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C5
3133/08	2008	Cat	amc, cef, NAL, ENR, CIP, LVX, NOR, kan, TOB, FFC, TET, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *aac(6')-Ib*, *tet(B)*, *sul1*, *sul2*, *dfrIa*	*fimH-1*, *entB*, *mrkD*, *ycfM*, *kfu*	ST15	C5

Supplementary Table 1 (continuation)- Characterisation of *K. pneumoniae* strains typed by MLST

Strain	Year	Species	Resistance phenotype[a]	Resistance genotype	Virulence genotype	ST	PFGE Cluster
4290/08	2008	Cat	amc, CEF, CTX, caz, CVN, fep, ATM, NAL, ENR, CIP, LVX, NOR, KAN, TOB, ffc, TET, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, tet(B), sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
4300/08	2008	Dog	amc, CEF, CTX, CAZ, CVN, fep, ATM, NAL, ENR, CIP, LVX, NOR, KAN, TOB, FFC, TET, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, tet(B), sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
5704A/08	2008	Cat	AMC, CEF, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, amk, GEN, KAN, NET, TOB, CHL, ffc, TET, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, tet(B), sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
5704B/08	2008	Cat	CEF, CTX, CAZ, CVN, fep, ATM, NAL, ENR, CIP, LVX, NOR, GEN, CHL, FFC	*bla$_{CTX-M-15}$*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
21/2011	2011	Dog	AMC, tzp, NAL, ENR, CIP, LVX, NOR, GEN, KAN, NET, TOB, CHL, ffc, tet, SXT	*bla$_{OXA-1}$, aac(6')-Ib*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
86/2015	2015	Cat	AMC, tzp, CEF, FOX, CTX, CAZ, CVN, fep, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, NET, TOB, CHL, ffc, NIT, TET, SXT	*bla$_{CMY-2}$, qnrB, aac(6')-Ib, sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu*	ST15	C5
GTAFD58A	2014	Human-CA	AMC, CEF, CTX, CVN, fep, atm, NAL, ENR, CIP, LVX, NOR	*bla$_{TEM}$, bla$_{CTX-M-15}$*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST15	C5
GTAN4020	2014	Human-CA	CEF, CTX, CAZ, CVN, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, TOB, CHL, FOF, TET, SXT	*bla$_{TEM-1}$, bla$_{SHV-28}$, qnrB, aac(6')-Ib, sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST15	S7
38/2015	2015	Cat	amc, CEF, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, NET, TOB, ffc, NIT, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, sul2*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST15	S8
72/2012KP	2012	Dog	AMC, TZP, CEF, FOX, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, KAN, TOB, NIT, TET	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, bla$_{CMY-2}$, aphAI-IAB, aac(6')-Ib, tet(A)*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST15	S10

Supplementary Table 1 (continuation)- Characterisation of *K. pneumoniae* strains typed by MLST

Strain	Year	Species	Resistance phenotype[a]	Resistance genotype	Virulence genotype	ST	PFGE Cluster
GTBJ307 Rosa	2014	Human-CA	CEF, CTX, CAZ, CVN, FEP, ATM, NIT	*bla*$_{CTX-M-15}$	*fimH-1, entB, mrkD, ycfM, kpn, YHPI*	ST29	C8
GTCJ3923	2014	Human-CA	amc, tzp, CEF, CTX, caz, CVN, FEP, ATM, nal, enr, cip, nor, GEN, KAN, TOB, CHL, ffc, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *qnrB, aac(6')-Ib, sul1, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST35	C8
PC28/15B	2015	Human-HA	AMC, TZP, CEF, CTX, CAZ, CVN, FEP, ATM, nal, enr, cip, GEN, kan, TOB, CHL, NIT, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *qnrB, aac(6')-Ib, sul1, sul2, dfrA12*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST35	C8
GTML38039	2014	Human-CA	AMC, TZP, CEF, FOX, CTX, CAZ, CVN, atm, FOF, NIT	*bla*$_{CMY-2}$	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST35	C3
4919/09	2009	Cat	AMC, TZP, CEF, FOX, CTX, CAZ, CVN, ATM, NAL, ENR, CIP, LVX, NOR, CHL, FFC, NIT, TET, SXT	*bla*$_{TEM}$, *bla*$_{CMY-2}$, *floR, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kpn*	ST37	S33
GTPD2025	2014	Human-CA	amc, CEF, CTX, CVN, fep, ATM, nal, enr, cip, nor, GEN, KAN, TOB, nit, TET, SXT	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *qnrB, aac(6')-Ib, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kfu*	ST42	S35
GTMR923	2014	Human-CA	AMC, tzp, CEF, CTX, CAZ, CVN, FEP, ATM, nal, ENR, CIP, lvx, NOR, GEN, KAN, NET, TOB, NIT, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *qnrB, aac(6')-Ib, sul2*	*fimH-1, entB, mrkD, ycfM, kpn, YHPI*	ST45	C15
GTLS335	2014	Human-CA	amc, CEF, CTX, caz, CVN, FEP, ATM, nal, enr, cip, GEN, KAN, TOB, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *qnrB, aac(6')-Ib, sul2*	*fimH-1, entB, mrkD, ycfM, kpn*	ST3031[b]	C15
5659/13	2013	Cat	amc, CEF, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, NET, TOB, NIT, TET, SXT	*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kpn*	ST147	S24
870/10	2010	Dog	NAL, enr, cip, KAN, CHL, ffc, nit, SXT	*aphAI-IAB, sul1, sul2*	*fimH-1, entB, mrkD, ycfM, kfu, YPIH*	ST274	S26

Supplementary Table 1 (continuation)- Characterisation of *K. pneumoniae* strains typed by MLST

Strain	Year	Species	Resistance phenotype[a]	Resistance genotype	Virulence genotype	ST	PFGE Cluster
26/2015	2015	Dog	nit	-	*fimH-1, entB, mrkD, ycfM, kfu*	ST294	C34
Sisi/vet	2014	Cat	AMC, CEF, FOX, CTX, CAZ, CVN, nal, enr	*bla$_{TEM}$, bla$_{CMY-2}$, qnrB*	*fimH-1, entB, mrkD, ycfM, kpn*	ST348	C18
GTDN614	2014	Human-CA	amc, CEF, CTX, CAZ, CVN, FEP, ATM, nal, enr, cip, nor, GEN, KAN, TOB, nit, TET, SXT	*bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kpn, YHPI*	ST348	C18
PC25/15B	2015	Human-HA	amc, tzp, CEF, CTX, CAZ, CVN, FEP, ATM, GEN, kan, TOB, NIT, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, sul2*	*fimH-1, entB, mrkD, ycfM*	ST423	C17
GTET170	2014	Human-CA	amc, CEF, CTX, caz, CVN, FEP, ATM, nal, enr, cip, nor, KAN, TOB, ffc, TET, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, qnrB, aac(6')-Ib, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kpn*	ST648	C6
GTMR2019	2014	Human-CA	AMC, tzp, CEF, CTX, caz, CVN, fep, ATM, NAL, ENR, CIP, lvx, NOR, GEN, KAN, TOB, chl, FFC, FOF, NIT, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, qnrB, aac(6')-Ib, sul1, sul2*	*fimH-1, entB, mrkD, ycfM, kpn, YHPI*	ST1674	C13
GTML31905	2014	Human-CA	AMC, CEF, CTX, CVN, FEP, nal, ENR, CIP, nor, KAN, TOB, ffc, NIT, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, qnrB, aac(6')-Ib, sul2*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST 2620	S17
4898/03	2003	Dog	TET, nit	*tet(A)*	*fimH-1, entB, mrkD, ycfM, kfu, iutA*	ST3027[b]	C2
5942/07	2007	Dog	nit	-	*fimH-1, entB, mrkD, allS*	ST3028[b]	S36
6874/10	2010	Dog	NAL, enr, cip, ffc, NIT	-	*fimH-1, entB, mrkD, ycfM, kfu*	ST3029[b]	S41

Supplementary Table 1 (continuation)- Characterisation of *K. pneumoniae* strains typed by MLST

Strain	Year	Species	Resistance phenotype[a]	Resistance genotype	Virulence genotype	ST	PFGE Cluster
3919/11	2011	Dog	AMC, TZP, CEF, NIT	*bla$_{TEM}$,*	*fimH-1, entB, mrkD, ycfM, kfu*	ST3030[b]	C16
PC67/15B	2015	Human-HA	amc, tzp, CEF, CTX, CAZ, CVN, FEP, ATM, NAL, ENR, CIP, LVX, NOR, TOB, NIT, TET, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, qnrB, aac(6')-Ib, tet(A), sul2*	*fimH-1, entB, mrkD, ycfM, kpn*	ST3033[b]	C16
GTMR2457	2014	Human-CA	AMC, CEF, CTX, caz, CVN, fep, atm, GEN, KAN, TOB, ffc, nit, SXT	*bla$_{TEM}$, bla$_{OXA-1}$, bla$_{CTX-M-15}$, aac(6')-Ib, sul1, sul2, dfrIa*	*fimH-1, entB, mrkD, ycfM, kfu, YHPI*	ST3032[b]	C8

Legend: [a]Fully-resistance is presented in caps lock and the intermediate resistance is presented in small caps.
[b]STs first described in this study.
fimH-1, type-1 adhesin; *mrkD*, type-3 adhesin; *kpn*, FimH-like adhesin; *ycfM*, outer membrane lipoprotein; *entB*, enterobactin; *iutA*, aerobactin; *kfu*, iron transporter with phosphotransferase function; YHPI, yersiniabactin high-pathogenicity island; *traT*, serum resistance-associated outer membrane lipoprotein; *allS*, allantoin metabolism associated gene; AMC, amoxicillin/clavulanate; CEF, cephalothin, FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CVN, cefovecin; TZP, piperacillin/tazobactam; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; NAL, nalidixic acid; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin; LVX, levofloxacin; GEN, gentamicin; KAN, kanamycin; NET, netilmicin; TOB, tobramycin; AMK, amikacin; CHL, chloramphenicol; FFC, florfenicol; TET, tetracycline; FOF, fosfomycin; NIT, nitrofurantoin; SXT, trimethoprim/sulfamethoxazole; Human-HA, human hospital-patients with UTI; Human-CA, human community-acquired UTI.

Supplementary Table 2 - Frequency of MDR *K. pneumoniae* strains

Number of resistant categories[a]	Companion animal (Total *N*=25)	Human-HA (Total *N* =19)	Human-CA (Total *N* =57)
3	12.0% (*n*=3)	15.8% (*n*=3)	3.5% (*n*=2)
4	8.0% (*n*=2)	5.3% (*n*=1)	5.3% (*n*=3)
5	16.0% (*n*=4)	5.3% (*n*=1)	10.5% (*n*=6)
6	16.0% (*n*=4)	10.5% (*n*=2)	1.75% (*n*=1)
7	12.0% (*n*=3)	5.3% (*n*=1)	1.75% (*n*=1)
8	8.0% (*n*=2)	5.3% (*n*=1)	1.75% (*n*=1)
9	8.0% (*n*=2)	0% (*n*=0)	0% (*n*=0)
Total	80.0% (*n*=20)	47.4% (*n*=9)	24.6% (*n*=14)

Legend: [a]Antimicrobial categories were defined according to Magiorakos et al. (2012). Total *N*, total number of tested isolates; *n*, number of positive isolates; Human-HA, human hospital-patients with UTI; Human-CA, human community-acquired UTI.

Supplementary Table 3 - Characteristics of *K. oxytoca* strains

Strain	Year	Species	Resistance profile[a]	Resistance genes	Virulence profile	PFGE Cluster
FMV1489/04	2004	Cat	AMC, CEF, FOX, CTX, CAZ, CVN, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, NET, TOB, CHL, ffc, TET	*bla_{TEM}, bla_{DHA-1}, aphAI-IAB, aac(6')-Ib, tet(B), sul1*	*entB*	C1
FMV72/2012KO	2012	Dog	AMC, CEF, FOX, CTX, CAZ, CVN, ATM, NAL, ENR, CIP, LVX, NOR, GEN, KAN, TOB, CHL, ffc, TET	*bla_{TEM}, bla_{DHA-1}, aphAI-IAB, aac(6')-Ib, sul1*	*entB*	C1
GTLI2612	2014	Human-CA	-	-	*entB*	S1

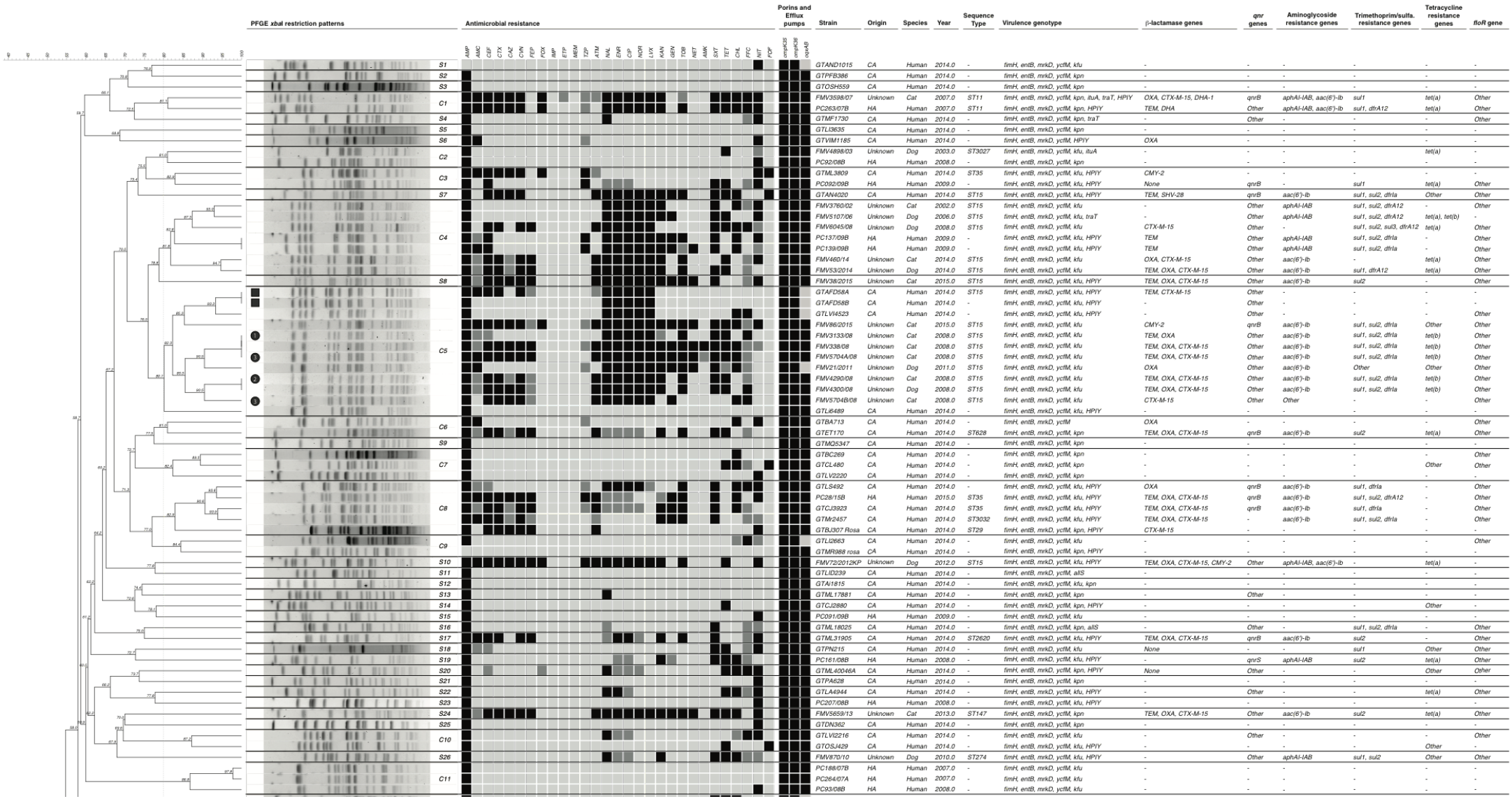
Legend: [a]Fully-resistance is presented in caps lock and the intermediate resistance is presented in small caps. *entB*, enterobactin; AMC, amoxicillin/clavulanate; CEF, cephalothin, FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CVN, cefovecin; TZP, piperacillin/tazobactam; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; NAL, nalidixic acid; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin; LVX, levofloxacin; GEN, gentamicin; KAN, kanamycin; NET, netilmicin; TOB, tobramycin; CHL, chloramphenicol; FFC, florfenicol; TET, tetracycline; NIT, nitrofurantoin; SXT, trimethoprim/sulfamethoxazole; Human-CA, human community-acquired UTI.

Supplementary Table 4 - Frequency of virulence genes in *K. pneumoniae* strains

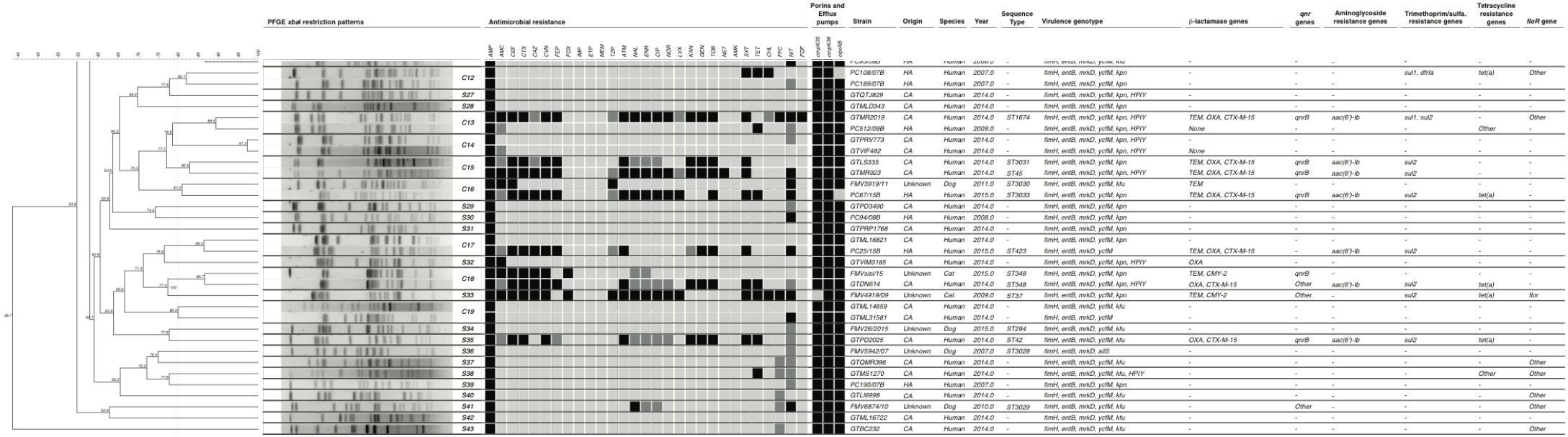
Target gene product/function	Target gene	Companion animal (Total *N*=25)	Human-HA (Total *N* =19)	Human-CA (Total *N* =57)	*P* value
Type-1 adhesin	*fimH-1*	100% (*n*=25)	100% (*n*=19)	100% (*n*=57)	1
Type-3 adhesin	*mrkD*	100% (*n*=25)	100% (*n*=19)	100% (*n*=57)	1
Outer membrane lipoprotein	*ycfM*	96.0% (*n*=24)	100% (*n*=19)	100% (*n*=57)	0.2475
FimH-like adhesin	*kpn*	16.0% (*n*=4)	42.1% (*n*=8)	52.6% (*n*=30)	0.0044
Catecholate-siderophore receptor	*iroN*	0% (*n*=0)	0% (*n*=0)	0% (*n*=0)	1
Enterobactin	*entB*	100% (*n*=25)	100% (*n*=19)	100% (*n*=57)	1
Aerobactin	*iutA*	8.0% (*n*=2)	0% (*n*=0)	0% (*n*=0)	0.0594
Iron transporter with phosphotransferase function	*kfu*	80.0% (*n*=20)	52.6% (*n*=10)	42.1% (*n*=24)	0.0025
Yersiniabactin high-pathogenicity island	*fyuA, irp-1, irp-2, ybtS*	16.0% (*n*=4)	42.1% (*n*=8)	43.9% (*n*=25)	0.0165
Serum resistance-associated outer membrane lipoprotein	*traT*	8.0% (*n*=2)	0% (*n*=0)	1.8% (*n*=1)	0.1506
Regulator of mucoid phenotype A	*rmpA*	0% (*n*=0)	0% (*n*=0)	0% (*n*=0)	1
Cytotoxic necrotizing factor-1	*cnf-1*	0% (*n*=0)	0% (*n*=0)	0% (*n*=0)	1
Allantoin metabolism associated gene	*allS*	4.0% (*n*=1)	0% (*n*=0)	3.5% (*n*=2)	1

Legend: Total *N*, total sample number; *n*, number of isolates; Human-HA, human hospital-patients with UTI; Human-CA, human community-acquired UTI.

Supplementary Figure 1 - PFGE patterns of chromosomal DNA restriction fragments from *K. pneumoniae* strains from companion animals (*n*= 26) and humans (*n*= 76) with UTI



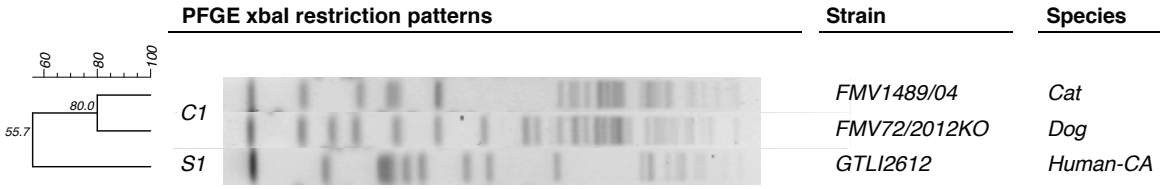
Supplementary Figure 1 (continuation)- PFGE patterns of chromosomal DNA restriction fragments from *K. pneumoniae* strains from companion animals (n= 26) and humans (n= 76) with UTI



	Strain	Origin	Species	Year	Sequence Type	Virulence genotype	β-lactamase genes	*qnr* genes	Aminoglycoside resistance genes	Trimethoprim/sulfa. resistance genes	Tetracycline resistance genes	*floR* gene
C12	PC108/07B	HA	Human	2007.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	*sul1, dfrIa*	*tet(a)*	*Other*
	PC189/07B	HA	Human	2007.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
S27	GTQTJ829	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn, HPIY*	-	-	-	-	-	-
S28	GTMLD343	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
C13	GTMR2019	CA	Human	2014.0	ST1674	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*TEM, OXA, CTX-M-15*	*qnrB*	*aac(6')-Ib*	*sul1, sul2*	-	*Other*
	PC512/09B	HA	Human	2009.0	-	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*None*	-	-	-	*Other*	-
C14	GTPRV773	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn, HPIY*	-	-	-	-	-	-
	GTVIF482	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*None*	-	-	-	-	-
C15	GTL5335	CA	Human	2014.0	ST3031	*fimH, entB, mrkD, ycfM, kpn*	*TEM, OXA, CTX-M-15*	*qnrB*	*aac(6')-Ib*	*sul2*	-	-
	GTMR923	CA	Human	2014.0	ST45	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*TEM, OXA, CTX-M-15*	*qnrB*	*aac(6')-Ib*	*sul2*	-	-
C16	FMV3919/11	Unknown	Dog	2011.0	ST3030	*fimH, entB, mrkD, ycfM, kfu*	*TEM*	-	-	-	-	-
	PC67/15B	HA	Human	2015.0	ST3033	*fimH, entB, mrkD, ycfM, kpn*	*TEM, OXA, CTX-M-15*	*qnrB*	*aac(6')-Ib*	*sul2*	*tet(a)*	-
S29	GTPD3490	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
S30	PC94/08B	HA	Human	2008.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
S31	GTPRP1768	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
C17	GTML16821	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
	PC25/15B	HA	Human	2015.0	ST423	*fimH, entB, mrkD, ycfM*	*TEM, OXA, CTX-M-15*	-	*aac(6')-Ib*	*sul2*	-	-
S32	GTVIM3185	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*OXA*	-	-	-	-	-
C18	FMVsisi/15	Unknown	Cat	2015.0	ST348	*fimH, entB, mrkD, ycfM, kpn*	*TEM, CMY-2*	*qnrB*	-	-	-	-
	GTDN614	CA	Human	2014.0	ST348	*fimH, entB, mrkD, ycfM, kpn, HPIY*	*OXA, CTX-M-15*	*Other*	*aac(6')-Ib*	*sul2*	*tet(a)*	-
S33	FMV4919/09	Unknown	Cat	2009.0	ST37	*fimH, entB, mrkD, ycfM, kpn*	*TEM, CMY-2*	*Other*	-	*sul2*	*tet(a)*	*flor*
C19	GTML14659	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	-
	GTML31581	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM*	-	-	-	-	-	-
S34	FMV26/2015	Unknown	Dog	2015.0	ST294	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	-
S35	GTPD2025	CA	Human	2014.0	ST42	*fimH, entB, mrkD, ycfM, kfu*	*OXA, CTX-M-15*	*qnrB*	*aac(6')-Ib*	*sul2*	*tet(a)*	-
S36	FMV5942/07	Unknown	Dog	2007.0	ST3028	*fimH, entB, mrkD, allS*	-	-	-	-	-	-
S37	GTQMR396	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	*Other*
S38	GTMS1270	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu, HPIY*	-	-	-	-	*Other*	*Other*
S39	PC190/07B	HA	Human	2007.0	-	*fimH, entB, mrkD, ycfM, kpn*	-	-	-	-	-	-
S40	GTLI6998	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	*Other*
S41	FMV6874/10	Unknown	Dog	2010.0	ST3029	*fimH, entB, mrkD, ycfM, kfu*	-	*Other*	-	-	-	*Other*
S42	GTML16722	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	-
S43	GTBC232	CA	Human	2014.0	-	*fimH, entB, mrkD, ycfM, kfu*	-	-	-	-	-	*Other*

Legend: Dashed line, cluster cut-off of 80%; Regarding PFGE: Grey circle, indicates urine samples collected from the same animal with two month intervals (total of three sample collections). The number inside the circle indicates the urine collection order; Grey square, indicates urine samples collected from the same human patient from the same urine sample; S, single pulse-type; C, pulse-type cluster; Regarding porins and efflux pumps: Black square, positive; Light grey square, negative; Regarding antimicrobials: Black square, resistant; Grey square, intermediate resistant; Light grey square, susceptible; AMP, ampicillin; AMC, amoxicillin/clavulanate; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; CVN, cefovecin; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; ETP, ertapenem; MEM, meropenem; TZP, piperacillin/tazobactam; ATM, aztreonam; NAL, nalidixic acid; ENR, enrofloxacin; CIP, ciprofloxacin; NOR, norfloxacin; LVX, levofloxacin; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; NET, netilmicin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; FFC, florfenicol; NIT, nitrofurantoin; FOF, fosfomycin; CA, community-acquired UTI; HA, hospital-patients with UTI; *fimH-1*, type-1 adhesin; *mrkD*, type-3 adhesin; *kpn*, FimH-like adhesin; *ycfM*, outer membrane lipoprotein; *entB*, enterobactin; *iutA*, aerobactin; *kfu*, iron transporter with phosphotransferase function; YHPI, yersiniabactin high-pathogenicity island; *traT*, serum resistance-associated outer membrane lipoprotein; *allS*, allantoin metabolism associated gene.

Supplementary Figure 2 - PFGE patterns of chromosomal DNA restriction fragments from *K. oxytoca* strains from companion animals (*n*= 2) and humans (*n*= 1) with UTI



Legend: Human-CA, community-acquired UTI; S, single pulse-type; C, pulse-type cluster.

4.2 Clonal relatedness of *Proteus mirabilis* strains causing urinary tract infections in companion animals and humans

Full paper published at the *Veterinary Microbiology*

Marques, C.*, Belas, A., Aboim, C., Trigueiro, G., Cavaco-Silva, P., Gama, L.T., Pomba, C. (2018). Clonal relatedness of *Proteus mirabilis* strains causing urinary tract infections in companion animals and humans. *Veterinary Microbiology,* 228, 77-82.

*The author performed the bacterial identification, antimicrobial susceptibility testing, DNA extraction and PFGE analysis of all *P. mirabilis* strains. The author conducted all PCR and sequencing assays except the *HpmB* screening. The author performed the data and statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on co-authors and reviewers revisions.

Partial results were presented as,

Three Poster communications at the international congress ASM Microbe 2016, 2016, Boston USA; at the international congress 27th ECCMID, 2017, Vienna, Austria; and at the international congress 28th ECCMID, 2018, Madrid, Spain.

Clonal relatedness of *Proteus mirabilis* strains causing urinary tract infections in companion animals and humans

Cátia Marques[a], Adriana Belas[a], Catarina Aboim[a], Graça Trigueiro[b], Patrícia Cavaco-Silva[c,d], Luís Telo Gama[a], Constança Pomba[a]

[a]Interdisciplinary Centre of Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal;
[b]Laboratório de Análises Clínicas Dr. Joaquim Chaves, Av. General Norton de Matos, 71 R/C, 1495-148 Algés, Portugal;
[c]Centro de Investigação Interdisciplinar Egas Moniz, Instituto Universitário Egas Moniz, Campus Universitário, Quinta da Granja, Monte de Caparica, 2829-511 Caparica, Portugal;
[d]Technophage, S.A., Avenida Prof. Egas Moniz, 1600-190 Lisboa, Portugal

Abstract

Proteus mirabilis is a major cause of urinary tract infection (UTI) in humans and companion animals. This study aimed to evaluate the antimicrobial resistance, virulence and clonal relatedness of *P. mirabilis* isolated from dogs, cats and humans with UTI.

P. mirabilis isolated from companion animals ($n = 107$) and humans ($n = 76$) with UTI were compared by PFGE analysis after overnight *Not*I macro-restriction using Dice/UPGMA with a 1.5% tolerance. Strains were characterised for antimicrobial resistance by disk diffusion. Twenty-four resistance genes and four virulence genes were screened by PCR.

Thirty-nine clusters (similarity >80%) and 73 single pulse-types were detected. Nine clusters included *P. mirabilis* isolated from community and hospital patients, including strains with 100% similarity. A high number of clusters (43.6%, $n = 17/39$) included strains from companion animals and humans. Similarity between some companion animal and human strains varied between 80–100%. One strain from a dog was 100% similar to one human community-acquired *P. mirabilis*. One *P. mirabilis* from a cat was found to be 94.7% and 92.4% similar to community and hospital patient strains, respectively. *P. mirabilis* CMY-2-producers did not cluster all together. Nevertheless, cluster C36 included five *P. mirabilis* from companion animals (similarity 85.8%–95.7%), of which, four (80%) were multidrug-resistant CMY-2-producers.

This study shows that companion animals and humans become infected with closely related *P. mirabilis* strains. The high number of clusters containing companion animals and human strains points to the zoonotic nature of *P. mirabilis*. These results underline the potential role of companion animals as reservoirs and in the dissemination of uropathogenic *P. mirabilis* to humans and vice versa.

Keywords: *Proteus mirabilis*, Urinary tract infection, Companion animal, Human, Clonal relatedness

Introduction

Proteus mirabilis are known to cause community-acquired urinary tract infections (UTI) in humans and are important agents of nosocomial UTIs (Armbruster & Mobley, 2012; Costa, Linhares, Ferreira, Neves & Almeida, 2018). After *E. coli*, which is the main cause of UTI, *P. mirabilis* is the second most common Enterobacteriaceae isolated in companion animals with UTI (Moyaert et al., 2017). The increasing antimicrobial resistance trends in bacteria isolated from companion animals creates concerns regarding the role of companion animals

as reservoirs of resistant bacteria to humans (Marques et al., 2018b; Pomba et al., 2017). CMY-2-producing *P. mirabilis*, which are resistant to third-generation cephalosporins and frequently multidrug-resistant (MDR), are increasingly being reported in companion animals in Portugal (Marques et al., 2018b). Since companion animals with UTI caused by *P. mirabilis* have high loads of bacteria in urine and faeces (Gaastra et al., 1996), the risk of dissemination of uropathogenic *P. mirabilis*, including potential resistant strains, into the owners living environment may be an important hazard.

Little is known about the clonal relatedness between human and companion animal uropathogenic *P. mirabilis*. Yet, this information is relevant to understand the role of companion animals and human as reservoirs of *P. mirabilis*. Only a limited number of European studies on resistance monitoring of UTI in companion animals are available (Marques et al., 2018b; Moyaert et al., 2017). Furthermore, only early studies report the frequency of virulence factors among *P. mirabilis* from companion animals (Bijlsma et al., 1995; Gaastra et al., 1996).

The main goal of this study was to determine the clonal relatedness of *P. mirabilis* isolated from companion animals (dogs and cats) and humans with UTI. Additionally, these uropathogenic *P. mirabilis* were characterised for the presence of antimicrobial resistance and virulence genes.

Material and methods

Bacteria isolates

This study included *P. mirabilis* isolated from humans with UTI ($n = 76$) at a private clinical analysis laboratory ($n = 50$) in 2014 and between 2006–2015 from hospital microbiology laboratories ($n = 26$) collecting samples from patients in the Lisbon area. The *P. mirabilis* isolated at the human private laboratory were obtained from humans with community-acquired UTI (CA-human). Samples from the hospital laboratories were obtained from hospital patients with UTI (HA-human) and were likely hospital-acquired. However, it was not possible to ensure that these infections were nosocomial due to insufficient patient data. Non-duplicate *P. mirabilis* strains from companion animals with UTI (dogs $n = 94$, cats $n = 13$) were obtained from 1999 to early 2015 at the Laboratory of Antimicrobial Resistance from the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine/University of Lisbon and therefore all companion animals were also from the Lisbon area.

Besides being collected from the same geographical region, there were no direct epidemiological relations between the sampled humans and companion animals. It was also not possible to obtain information regarding the overall daily contact of the sampled humans and companion animals with UTI. Moreover, data on prior antimicrobial use was not available.

Bacteriological methods

P. mirabilis strains were isolated by standard quantitative urine cultures and species identification was conducted by phenotypical tests (API20E and VITEKII, BioMérieux, Marcy-l'Étoile, France) and confirmed by *P. mirabilis* species-specific PCR (Marques et al., 2018b).

Susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion method according to CLSI guidelines (CLSI, 2017; CLSI, 2018). Isolates were tested for susceptibility against a total of twenty-six antimicrobials (Oxoid, Hampshire, UK): ampicillin 10µg, amoxicillin/clavulanate 30µg, cefoxitin 30µg, cefotaxime 30µg, ceftazidime 30µg, cefovecin 30µg, piperacillin/tazobactam 110µg, cefepime 30µg, imipenem 10µg, meropenem 10µg, aztreonam 30µg, ertapenem 10µg, nalidixic acid 30µg, ciprofloxacin 5µg, enrofloxacin 5µg, norfloxacin 10µg, levofloxacin 5µg, gentamicin 10µg, kanamycin 30µg, netilmicin 30µg, tobramycin 10µg, amikacin 30µg, chloramphenicol 30µg, florfenicol 30µg, fosfomycin 50µg and trimethoprim/sulfamethoxazole 25µg.

For cefovecin and enrofloxacin results interpretation, the veterinary CLSI breakpoints were applied (CLSI, 2018). Florfenicol (a fluorinated compound in use for bovine respiratory infections) was tested for epidemiological purposes only. Since there are no defined florfenicol breakpoints for companion animals nor humans, interpretation diameter limits were derived from the veterinary CLSI breakpoints for swine infections (CLSI, 2018). The Societé Française de Microbiology breakpoints were used for fosfomycin (SFM, 2010). Since gentamicin breakpoints only vary between veterinary and human CLSI guidelines by 1 mm in the susceptible/intermediate categorisation (CLSI, 2017; CLSI, 2018), human CLSI was used. The remaining antimicrobials were interpreted according to human CLSI breakpoints (CLSI, 2017), since breakpoints either are equal or are not determined in veterinary CLSI standards.

Antimicrobial categories were used to characterise MDR Enterobacteriaceae (Magiorakos et al., 2012). *P. mirabilis* were considered as MDR when resistant to three or more antimicrobial categories.

Antimicrobial resistance determinants

Ampicillin-resistant/intermediate isolates were tested for the presence of bla_{TEM}, bla_{SHV} and bla_{OXA-1} β-lactamase genes by PCR (Pomba et al., 2006). The presence of ESBL ($bla_{CTX-M-type}$) and AmpC ($bla_{CMY-type}$, $bla_{LAT-type}$, bla_{BIT-1}, $bla_{DHA-type}$, $bla_{MOX-type}$, $bla_{ACT-type}$, $bla_{FOX-type}$ and $bla_{MIR-type}$) β-lactamase genes among third-generation cephalosporin-resistant strains has been characterised elsewhere (Marques et al., 2018b).

Antimicrobial-resistant/intermediate isolates were further screened by PCR for the presence of antimicrobial resistance genes against aminoglycosides [*aphAI-IAB*, *aac(6')-Ib* and *aac(3')-IV*] (Frana et al., 2001; Sáenz et al., 2004; Robicsek et al., 2006a), phenicols (*floR*) (Sáenz et

al., 2004), fluoroquinolones (*qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*) (Robicsek et al., 2006b; Cavaco et al., 2009; Wang et al., 2009) and trimethoprim/sulfamethoxazole (*sul1*, *sul2*, *sul3*, *dfrA12* and *dfrIa* [targeting *dfrA1*, *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*]) (Sáenz et al., 2004; Guerra et al., 2001; Maynard et al., 2003; Perreten & Boerlin, 2003).

Virulence genes

All *P. mirabilis* were screened by PCR for the presence of *ucaA* (urothelial cell adhesion fimbriae [UCA/NAF]), *mrpA* (mannose-resistant *Proteus*-like fimbriae [MR/P]) and *pmfA* (*Proteus mirabilis* fimbriae [PMF]) fimbriae genes and for the presence of haemolysin *hpmA*/*hpmB* codifying genes (Sosa et al., 2006; Cestari et al., 2013).

Population structure and data analysis

P. mirabilis were characterised by overnight restriction with *Not*I (Thermo Scientific™, Lisbon, Portugal) and subsequent PFGE on a CHEF DR II-apparatus (Biorad, Hercules, CA, USA). The restriction fragments were resolved in a 1% agarose gel (agarose pulse-field grade, Nzytech - Genes and Enzymes, Lisbon, Portugal) using the following previously described block conditions: 1–30 s for 8 h followed by 30–70 s for 16 h at 14 °C, 6 V/cm2 (Sabbuba et al., 2003).

*P. mirabilis Not*I restriction PFGE-pattern analysis was conducted with the Bionumerics software, version 6.6, (Applied Maths from BioMérieux, Marcy-l'Étoile, France) using Dice/UPGMA clustering method with a tolerance of 1.5% and a clustering cut-off of 80%.

The SAS statistical software package for Windows, version 9.3, (SAS Institute Inc, Cary, North Carolina, USA) was used to perform statistical analysis. Comparisons between groups were conducted by Fisher's exact test, with an alpha value of 0.05.

Results

Antimicrobial resistance and antimicrobial resistance determinants

Considering β-lactam non-susceptible strains (categorised as antimicrobial resistant or -intermediate isolates), *P. mirabilis* from companion animals and CA-humans were frequently resistant only to ampicillin (46.9%, n= 15/32 and 86.7%, n = 13/15, respectively) while HA-human *P. mirabilis* were frequently resistant to ampicillin and intermediate or resistant to amoxicillin/clavulanate (61.5%, n= 8/13) (Table 1).

β-lactam non-susceptible *P. mirabilis* strains frequently harboured bla_{TEM} (88.3%) while bla_{OXA-1} and bla_{SHV} were not detected (Table 2). Third-generation cephalosporin-resistance was only present in *P. mirabilis* from companion animals and was mostly linked to the presence of the bla_{CMY-2} gene.

Table 1 - Antimicrobial resistance frequencies of companion animal and human *P. mirabilis* strains

Antimicrobials	Companion animal (*N* = 107)		CA-human (*N* = 50)		HA-human (*N* = 26)	
	%R	%I	%R	%I	%R	%I
Ampicillin	28.0% (*n* = 30)	1.9% (*n* = 2)	30.0% (*n* = 15)	0% (*n* = 0)	50.0% (*n* = 13)	0% (*n* = 0)
Amoxicillin/clavulanate	9.4% (*n* = 10)	5.6% (*n* = 6)	2.0% (*n* = 1)	2.0% (*n* = 1)	3.8% (*n* = 1)	26.9% (*n* = 7)
Cefoxitin	9.4% (*n* = 10)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Cefotaxime	9.4% (*n* = 10)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Ceftazidime	5.6% (*n* = 6)	3.7% (*n* = 4)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Cefovecin	9.4% (*n* = 10)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Piperacillin/tazobactam	1.9% (*n* = 2)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Cefepime	0% (*n* = 0)	5.6% (*n* = 6)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Imipenem	0% (*n* = 0)	7.5% (*n* = 8)	0% (*n* = 0)	2% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)
Meropenem	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Aztreonam	1.9% (*n* = 2)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Ertapenem	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Nalidixic acid	26.2% (*n* = 28)	4.7% (*n* = 5)	16.0% (*n* = 8)	4.0% (*n* = 2)	42.3% (*n* = 11)	0% (*n* = 0)
Ciprofloxacin	8.4% (*n* = 9)	5.6% (*n* = 6)	8.0% (*n* = 4)	2.0% (*n* = 1)	26.9% (*n* = 7)	3.8% (*n* = 1)
Enrofloxacin	26.2% (*n* = 28)	1.9% (*n* = 2)	16.0% (*n* = 8)	0% (*n* = 0)	42.3% (*n* = 11)	0% (*n* = 0)
Norfloxacin	5.6% (*n* = 6)	1.9% (*n* = 2)	6.0% (*n* = 3)	0% (*n* = 0)	19.2% (*n* = 5)	3.8% (*n* = 1)
Levofloxacin	7.5% (*n* = 8)	0% (*n* = 0)	8.0% (*n* = 4)	0% (*n* = 0)	23.1% (*n* = 6)	0% (*n* = 0)
Gentamicin	6.5% (*n* = 7)	2.8% (*n* = 3)	10.0% (*n* = 5)	0% (*n* = 0)	30.8% (*n* = 8)	0% (*n* = 0)
Kanamycin	20.6% (*n* = 22)	5.6% (*n* = 6)	12.0% (*n* = 6)	2.0% (*n* = 1)	11.5% (*n* = 3)	0% (*n* = 0)
Netilmicin	3.7% (*n* = 4)	2.8% (*n* = 3)	2.0% (*n* = 1)	0% (*n* = 0)	7.7% (*n* = 2)	0% (*n* = 0)
Tobramycin	5.6% (*n* = 6)	1.9% (*n* = 2)	4.0% (*n* = 2)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Amikacin	2.8% (*n* = 3)	0.9% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)
Trimethoprim/sulfa.	35.5% (*n* = 38)	1.9% (*n* = 2)	26.0% (*n* = 13)	0% (*n* = 0)	38.5% (*n* = 10)	3.8% (*n* = 1)
Chloramphenicol	28.0% (*n* = 30)	18.7% (*n* = 20)	20.0% (*n* = 10)	8.0% (*n* = 4)	11.5% (*n* = 3)	11.5% (*n* = 3)
Florfenicol	5.6% (*n* = 6)	10.3% (*n* = 11)	4.0% (*n* = 2)	2.0% (*n* = 1)	0% (*n* = 0)	15.4% (*n* = 4)
Fosfomycin	5.6% (*n* = 6)	0% (*n* = 0)	2.0% (*n* = 1)	0% (*n* = 0)	0% (*n* = 0)	0% (*n* = 0)

Legend: *N*, total sample number; %R, percentage of resistant isolates; %I percentage of intermediate isolates; *n*, number of isolates; HA-human, human hospital-patient UTI; CA-human, human community-acquired UTI.

Resistance to fluoroquinolones was higher in isolates from HA-human UTIs, followed by companion animal and CA-Human UTIs (Table 1). Three main fluoroquinolone-non-susceptible phenotypes were detected, namely non-susceptibility to nalidixic acid and enrofloxacin with or without ciprofloxacin-non-susceptibility and non-susceptibility to all tested fluoroquinolones including norfloxacin and levofloxacin. Interestingly, the non-susceptibility to nalidixic acid and enrofloxacin phenotype was significantly more common in companion animal *P. mirabilis* strains isolated prior to 2006 (75%, $n = 12/16$, $P = 0.0003$) while non-susceptibility to all fluoroquinolones became significantly more frequent thereafter (44.4%, $n = 8/18$,

P = 0.0031) (data not shown). Only *qnrD* was detected in *P. mirabilis* from companion animals and humans (Table 2).

Table 2- Antimicrobial resistance genes detected in antimicrobial non-susceptible *P. mirabilis* strains

Antimicrobial	Gene	Overall	Companion animal	CA-human	HA-human	*P* value[a]
β-lactams	*bla*$_{TEM}$	88.3% (*n* = 53/60)	84.4% (*n* = 27/32)	86.7% (*n* = 13/15)	100% (*n* = 13/13)	0.4320
Third-generation cephalosporins	*bla*$_{CMY-2}$	90.0% (*n* = 9/10)	90.0% (*n* = 9/10)	–	–	–
	bla$_{DHA-1}$	10.0% (*n* = 1/10)	10.0% (*n* = 1/10)	–	–	–
Fluoroquinolones	*qnrD*	16.4% (*n* = 9/55)	17.6% (*n* = 6/34)	30% (*n* = 3/10)	0% (*n* = 0/11)	1
Aminoglycosides	*aphAI-IAB*	56.2% (*n* = 27/48)	65.5% (*n* = 19/29)	55.6% (*n* = 5/9)	30.0% (*n* = 3/10)	0.1426
	aac(3')-IV	2.1% (*n* = 1/48)	3.4% (*n* = 1/29)	0% (*n* = 0/9)	0% (*n* = 0/10)	1
Folate pathway inhibitors	*sul1*	29.7% (*n* = 19/64)	17.5% (*n* = 7/40)	38.5% (*n* = 5/13)	63.6% (*n* = 7/11)	0.0102
	sul2	46.9% (*n* = 30/64)	50.0% (*n* = 20/40)	61.5% (*n* = 8/13)	18.2% (*n* = 2/11)	0.6085
	sul3	1.6% (*n* = 1/64)	2.5% (*n* = 1/40)	0% (*n* = 0/13)	0% (*n* = 0/11)	1
	dfrIa	81.2% (*n* = 52/64)	85.0% (*n* = 34/40)	69.2% (*n* = 9/13)	81.8% (*n* = 9/11)	0.3414
Phenicols	*floR*	6.8% (*n* = 5/74)	9.8% (*n* = 5/51)	0% (*n* = 0/15)	0% (*n* = 0/8)	0.3161

Legend: *n*, number of positive among tested isolates; HA-human, human hospital-patient UTI; CA-human, human community-acquired UTI; -, no strains were tested since all strains were susceptible; [a]*P. mirabilis* from companion animals were compared with *P. mirabilis* from humans (CA-human+HA-human) by Fisher's exact test.

Among aminoglycosides, kanamycin and gentamicin showed the highest non-susceptibility frequencies (Table 1) and the *aphAI-IAB* was the most common resistance gene detected (Table 2).

Both in companion animal and human *P. mirabilis*, resistance against trimethoprim/sulfamethoxazole was high (26.0%–38.5%) (Table 1). The frequency of *sul1* was significantly lower in *P. mirabilis* from companion animals when compared to strains from humans overall (Table 2). However, when compared to strains from CA-human only, a statistical significant difference was not detected (*P* = 0.1403). The frequency of *sul2* in *P. mirabilis* from CA-human was significantly higher than in strains from HA-human (*P* = 0.0470). Nonetheless, *sul1* and *sul2* were detected in *P. mirabilis* from all patients while *sul3* was only detected in *P. mirabilis* from companion animals (Table 2). All trimethoprim/sulfamethoxazole non-susceptible *P. mirabilis* lacked *dfrA12* and several strains were negative for *sul1*, *sul2* and *sul3* (companion animal 37.5%, *n* = 15/40; CA-human 15.4%,

n = 2/13; HA-human 18.2%, *n* = 2/11). Overall, *dfrIa* was common in *P. mirabilis* from all patients (Table 2).

Chloramphenicol-resistance was high, especially in companion animal and CA-human *P. mirabilis* (Table 1). The *floR* gene was only detected in *P. mirabilis* from companion animals (Table 2) and all were co-resistant to chloramphenicol and florfenicol.

MDR *P. mirabilis* were more frequent among HA-human (42.3%, *n* = 11/26) strains, followed by companion animal (21.5%, *n* = 23/107) and CA-human (18%, *n* = 9/50). However, most MDR *P. mirabilis* were only resistant to three antimicrobial categories (HA-human, 30.8%, *n* = 8/26; CA-human, 10%, *n* = 5\50 and companion animals, 10.3%, *n* = 11/107). *P. mirabilis* from human patients were resistant to up to 5 antimicrobial categories while third-generation cephalosporin-resistant *P. mirabilis* from companion animals were resistant to 5 up to 9 antimicrobial categories.

Virulence genes

All fimbriae genes (*ucaA*, *pmfA* and *mrpA*) were highly frequent among *P. mirabilis* isolated from companion animals and humans (Table 3), nevertheless, *ucaA* gene was less frequent (Table 3). The haemolysin *HmpA*/*HmpB* genes were present in all tested *P. mirabilis* (Table 3).

Table 3- Companion animal and human *P. mirabilis* strains virulence genes

Virulence genes	Overall (*N*=183)	Companion animal (*N*=107)	CA-human (*N*=50)	HA-human (*N*=26)	*P* value[a]
Fimbriae					
mrpA	98.9% (*n*=181)	99.1% (*n*=106)	98% (*n*=49)	100% (*n*=26)	1
pmfA	98.9% (*n*=181)	100% (*n*=107)	96% (*n*=48)	100% (*n*=26)	0.1711
ucaA	85.2% (*n*=156)	86.9% (*n*=93)	80% (*n*=40)	88.5% (*n*=23)	0.5272
Haemolysin					
HmpA/HmpB	100% (*n*=183)	100% (*n*=107)	100% (*n*=50)	100% (*n*=26)	1

Legend: *n*, number of isolates; HA-human, human hospital-patient UTI; CA-human, human community-acquired UTI;
[a]*P. mirabilis* from companion animals were compared with *P. mirabilis* from humans (CA-human+HA-human) by Fisher's exact test.

Population structure

Thirty-nine clusters and 73 unique pulse-types were detected (Figure S1). Nine clusters included *P. mirabilis* from both CA-human and HA-human UTIs (C2, C3, C8, C10, C13, C24, C26, C28 and C29) with some including highly related (Dice index 90%–100%) CA-human and HA-human *P. mirabilis* strains (C2, C10, C13, C24, C26 and C28). It should be noted that some of these strains report to different time periods and were obtained from unrelated patients.

A high number of clusters (43.6% *n* = 17/39) included *P. mirabilis* from companion animals and humans (C2, C3, C6, C13, C14, C15, C16, C17, C22, C25, C26, C27, C28, C31, C37, C38 and C39). The clonal relatedness between companion animal and human *P. mirabilis* varied between 80–100%. Several companion animal strains included in these clusters were isolated from different time periods. Cluster C28 is noteworthy since it includes one strain, from a dog, 100% similar to one CA-human *P. mirabilis*. Furthermore, one *P. mirabilis* from a cat (C2) was also found to be 94.7% and 92.4% similar to one CA-human and HA-human strain, respectively. Some *P. mirabilis* strains isolated from dogs and cats (C21 and C34) also showed high similarity (90%–100%). Based on veterinary records, only strains FMV5954B/03 and FMV5955/03 were found to be epidemiologically related, since they were isolated in the same week from two cats admitted at the same veterinary practice.

P. mirabilis CMY-2-producer strains from companion animals did not cluster all together. However, C36 included five *P. mirabilis* from companion animals (85.8%–95.7% similarity), of which, four (80%) were multidrug-resistant CMY-2-producers.

Discussion

Currently there are few studies characterising the clonal population structure epidemiology of human or companion animal uropathogenic *P. mirabilis* strains (Gaastra et al., 1996; Sabbuba et al., 2003; Mathur et al., 2008). A high number of single pulse-types were detected both in human and in companion animal *P. mirabilis* strains in this study. Nevertheless, several PFGE clusters composed of *P. mirabilis* isolated in different years were detected, pointing to a higher dissemination of some clusters.

P. mirabilis is known to cause nosocomial infections (Armbruster & Mobley, 2012). The detection of several closely related HA-human strains likely resulted from nosocomial dissemination. The two identical *P. mirabilis* from two cats admitted at the same clinic, may also point to nosocomial events. Notably, the nine clusters that included *P. mirabilis* from CA-human and HA-human UTIs highlight the possible circulation of some strains between the hospital and community environment.

One previous study conducted on a limited number of *P. mirabilis* of several sources, detected one *P. mirabilis* strain from a dog that was closely related to one strain from a human (Schultz et al., 2015). In the present study, a high number of PFGE clusters (43.6%) contained strains from companion animal and human origin, including some with significant similarity (90%–100%). Thus, these results point to the zoonotic potential of uropathogenic *P. mirabilis*. The close similarity of some strains from cats and dogs also highlights the possibility of *P. mirabilis* transfer between pets.

Overall, HA-human *P. mirabilis* seemed to have higher antimicrobial resistance frequencies. However, the fact that this study relied on convenience samples collected in different time-

spans is a study limitation that restricts more extensive comparisons. Another limitation from this study was the lack of data regarding any prior antimicrobial treatment to the sampling.

Nonetheless, some of the higher resistance frequencies in this study were detected against important antimicrobials for UTI treatment, such as trimethoprim/sulfamethoxazole, fluoroquinolones and β-lactams. A previous study from Portugal reported higher antimicrobial resistance frequencies among *P. mirabilis* from humans with community-acquired UTI (Costa et al., 2018). Part of the companion animal *P. mirabilis* strains here characterised have been included in a previous study analysing the temporal trends of antimicrobial resistance in UTI (Marques et al., 2018b). It is noteworthy that in this previous study, a significant increase in MDR *P. mirabilis* third-generation cephalosporin-resistance was detected due to the presence of CMY-2 β-lactamase (Marques et al., 2018b).

The *P. mirabilis* isolated from humans in this study were all third-generation cephalosporin-susceptible. However, the detection of AmpC-producing *P. mirabilis* from human infection is being increasingly reported in Europe (D'Andrea et al., 2011; Aogáin et al., 2016). Interestingly, in our study, several MDR CMY-2-producing *P. mirabilis* strains from companion animals, including unrelated strains from a wide time frame, clustered together. This may point to the expansion of MDR CMY-2-producing PFGE C36 among companion animals. A previous study conducted in the UK, using whole genome sequencing (WGS), has reported the expansion of two clones of CMY-2-producing *P. mirabilis* in human infection (Aogáin et al., 2016). Since all CMY-2-producing *P. mirabilis* from companion animals from the present study were MDR and resistant to 5–9 antimicrobial categories, their possible expansion is worrisome.

Companion animal and human *P. mirabilis* strains harboured similar antimicrobial resistance genes except bla_{CMY-2}, *sul3*, *floR* and *aac(3)-IV*, only detected in companion animals. Therefore, *P. mirabilis* from companion animals were reservoirs of important resistance genes. The high dissemination of *sul1* and *sul2* in *P. mirabilis* is in line with the high frequency of these resistance genes in other Enterobacteriaceae from Portugal (Antunes, Machado, Sousa & Peixe, 2005). The trimethoprim/sulfamethoxazole non-susceptible strains lacking *sul1*, *sul2* and *sul3* may harbour untested resistance mechanism such as the recently described *sul4* (Razavi et al., 2017). The presence of *qnrD* among fluoroquinolone-non-susceptible *P. mirabilis* agrees with previous published data (Harada et al., 2014; Jacoby et al., 2014; Schultz et al., 2017a; de Jong et al., 2018). Nevertheless, the frequency of *qnrD* detected in *P. mirabilis* from companion animals in the present study was higher than in previous studies (Harada et al., 2014; Schultz et al., 2017a). The main acquired fluoroquinolone-resistance mechanism in Enterobacteriaceae is the acquisition of mutations on the quinolone resistance-determining regions (QRDR) (Weigel et al., 2002; Harada et al., 2014). Furthermore, generally resistance to a given fluoroquinolone is accompanied by elevated non-wild type MIC values of other fluoroquinolones (Hernández, Martínez-Martínez, Pascual, Suárez & Perea, 2000). It was interesting to notice that several *P. mirabilis* strains from the present study did not have

clinical cross-resistance to all tested fluoroquinolones. The higher clinical resistance of *P. mirabilis* towards enrofloxacin than to ciprofloxacin was also noted in a previous study conducted in *P. mirabilis* of dog origin and several QRDR mutations were reported (Harada et al., 2014). QRDR mutations of ciprofloxacin-resistant and levofloxacin-susceptible *P. mirabilis* strains from humans have also been reported (Weigel et al., 2002). The temporal change in fluoroquinolone-resistance phenotype in *P. mirabilis* from companion animals could be a consequence of antimicrobial use over time. The characterisation of QRDR and determination of fluoroquinolone minimal inhibitory concentrations should be pursued in the future.

The *aphAI-IAB* aminoglycoside phosphotransferase has been previously detected in *P. mirabilis* through WGS (Di Pilato et al., 2016). To our knowledge, this is the first detection of *aphAI-IAB* in *P. mirabilis* from companion animals and the first report of its frequency among clinical relevant *P. mirabilis*. The detection of *floR* only in companion animal strains arguments to different chloramphenicol/florfenicol-resistance epidemiology between companion animals and humans. In *Salmonella*, the *floR* is sometimes present in the *Salmonella* genomic island 1 (SGI1) (Hall, 2010). Interestingly, some SGI1 variants have already been described in MDR *P. mirabilis* from companion animals and humans (Schultz et al., 2015).

The high frequency of *ucaA*, *mrpA* and *pmfA* fimbriae genes detected in this study could be expected based on previous phenotypical and genotypic studies conducted in *P. mirabilis* from companion animals and humans, respectively (Bijlsma et al., 1995; Gaastra et al., 1996; Sosa et al., 2006). The comparison of UCA/NAF, MR/P and PMF fimbrial adhesins among strains from companion animals and humans in the future may help to better understand *P. mirabilis* zoonotic potential. Although, early phenotypical studies have shown that most *P. mirabilis* from dogs with UTI were haemolytic (Gaastra et al., 1996), to our knowledge, this is the first report of the haemolysin *HmpA*/*HmpB* coding genes presence and frequency among companion animal strains. The high frequency of *HmpA*/*HmpB* in *P. mirabilis* from humans agrees with previous results (Cestari et al., 2013).

There is a lack of studies comparing the clonal relatedness between *P. mirabilis* from companion animals and humans. Furthermore, the few existing studies are focussed in selected resistant population from several infection sources (Schultz et al., 2015; Schultz et al., 2017b). To our best knowledge, this is the first study comparing the clonal relatedness of a large number of *P. mirabilis* obtained exclusively from humans and companion animals with UTIs.

In this study, the *P. mirabilis* clonal relatedness was accessed only by PFGE analysis and not also by MLST because the latter isn't available for this bacterial specie. Future studies using whole genome sequencing could help to overcome this limitation and further contribute to understand the risk of uropathogenic *P. mirabilis* animal-human transfer.

Studies conducted on *E. coli*, have found that companion animals and humans may share uropathogenic strains within the same household (Johnson & Clabots, 2006). Similar studies

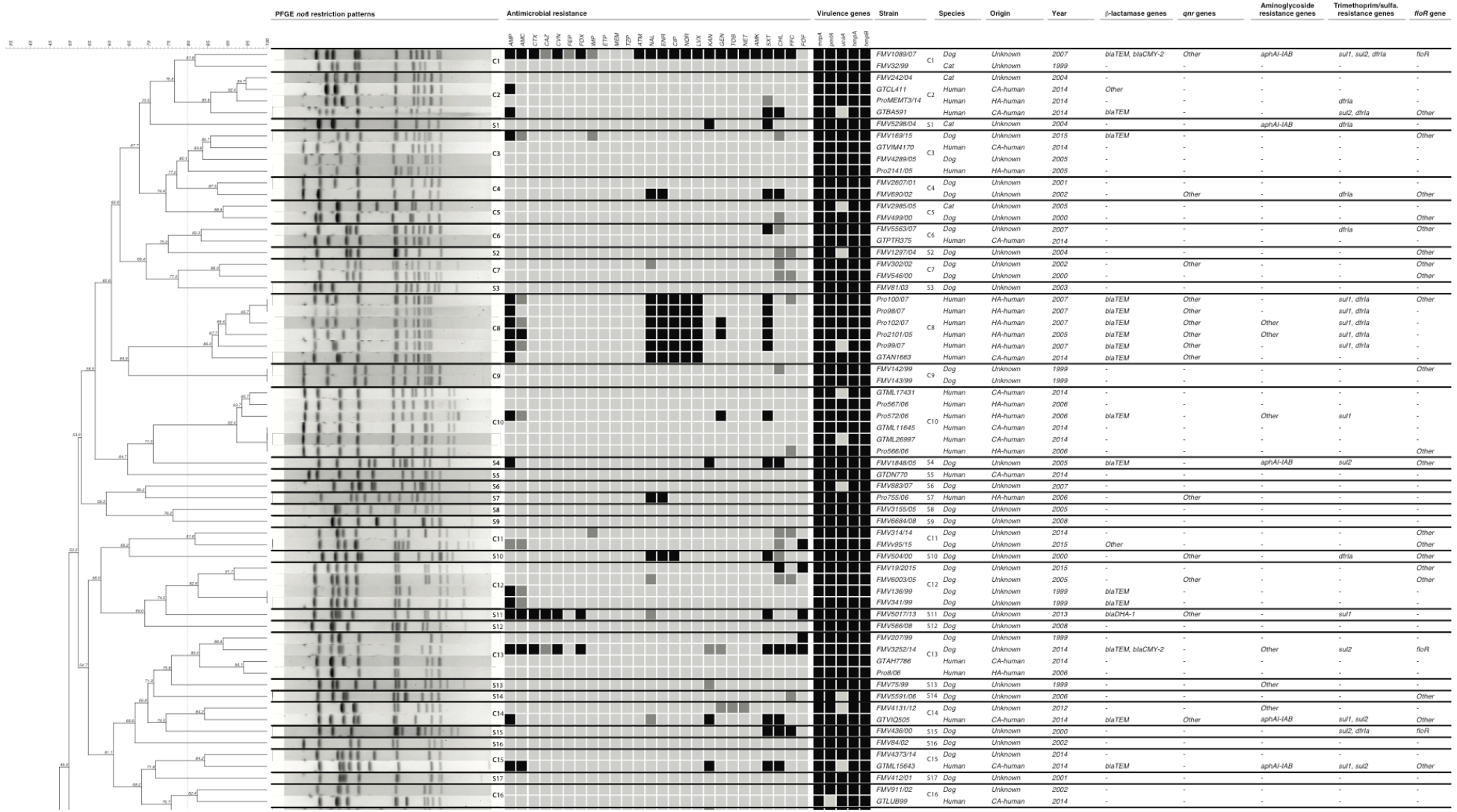
should be conducted regarding *P. mirabilis* to fully evaluate the risk of animal-human transfer during UTI. Nevertheless, considering the clonal relatedness between *P. mirabilis* from companion animals and humans in this study, the role of companion animals in the dissemination of uropathogenic resistant *P. mirabilis* should not be neglected. The route of transfer (human to animal and vice versa) is also unknown and warrants attention in future studies. The lack of hygiene practices has been pointed as a factor for dissemination of *P. mirabilis* (Drzewiecka, 2016) and therefore should be considered when handling companion animals.

Conclusion

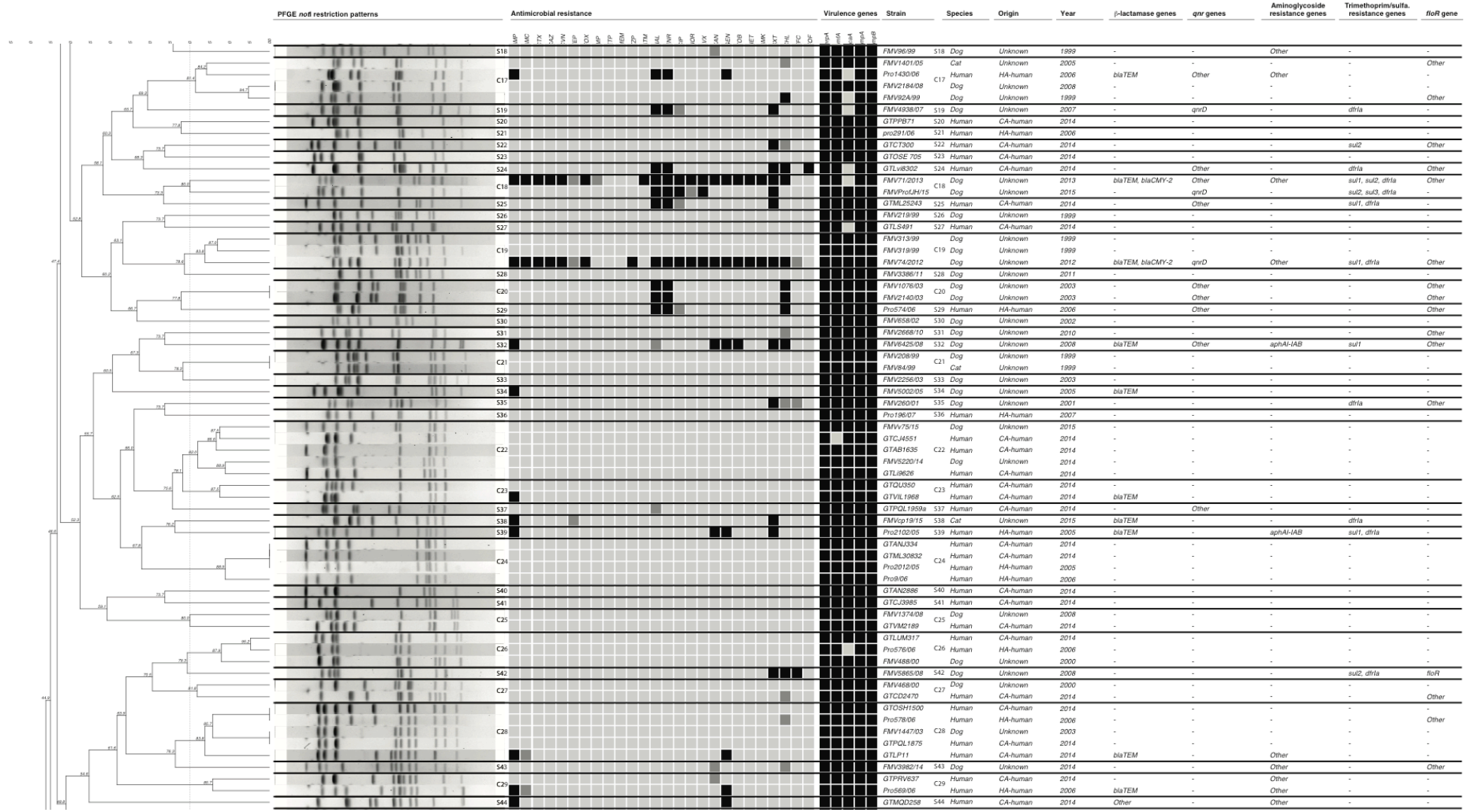
This study shows that UTI in companion animals and humans may be caused by closely related *P. mirabilis* strains and that *P. mirabilis* from both origins have common antimicrobial resistant and virulence genes. Taken together, these results underline the possible zoonotic nature of *P. mirabilis* causing UTIs in companion animals, which has important public-health implications.

Pending additional research, companion animals might be regarded as possible sources of uropathogenic *P. mirabilis* to humans and other companion animals living in close contact.

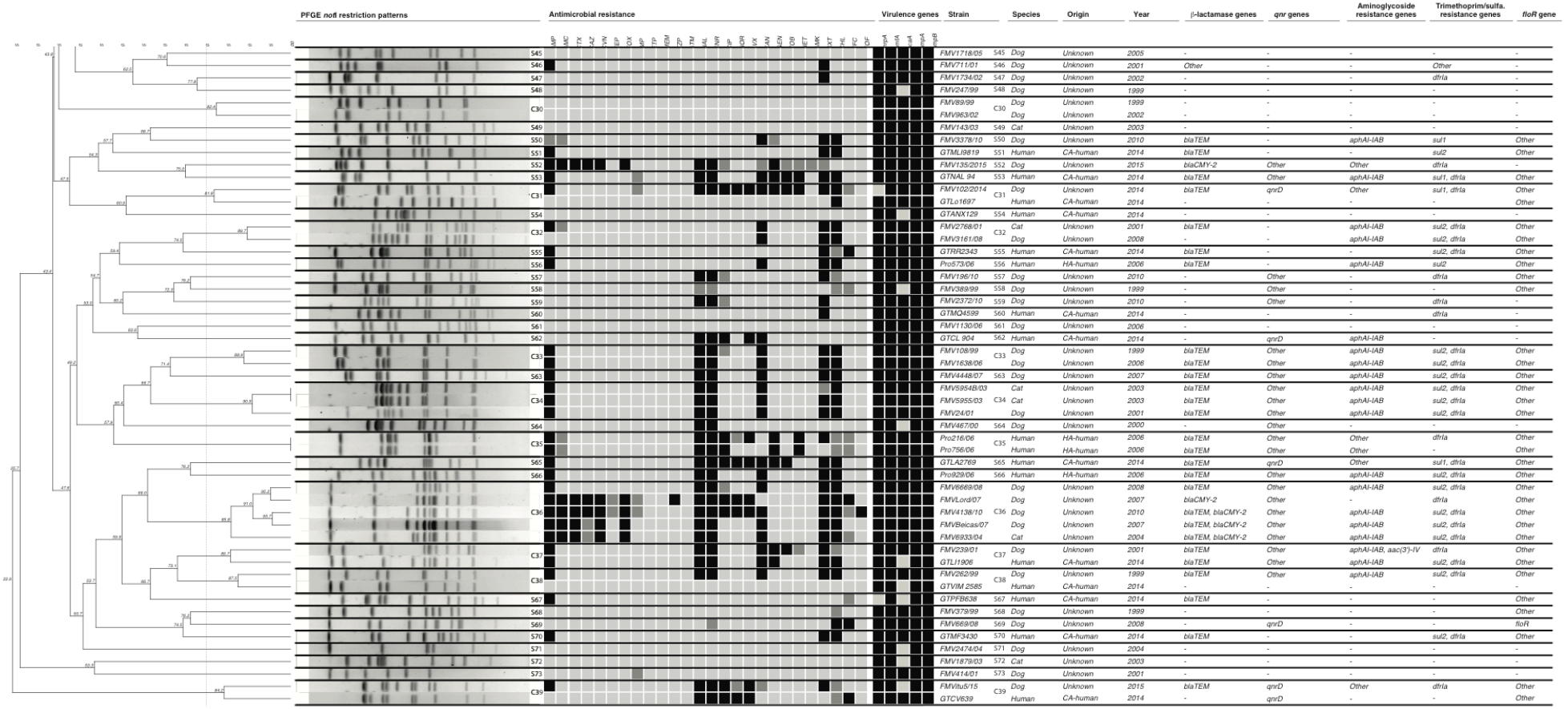
Supplementary Figure 1 - PFGE patterns of chromosomal DNA restriction fragments from *P. mirabilis* strains from companion animals and humans with UTI



Supplementary Figure 1 (continuation)- PFGE patterns of chromosomal DNA restriction fragments from *P. mirabilis* strains from companion animals and humans with UTI



Supplementary Figure 1 (continuation)- PFGE patterns of chromosomal DNA restriction fragments from *P. mirabilis* strains from companion animals and humans with UTI



Legend: Dashed line, cluster cut-off of 80%; Regarding virulence genes: Black square, positive; Light grey square, negative; Regarding antimicrobials: Black square, resistant; Grey square, intermediate resistant; Light grey square, susceptible; S, Single pulse-type; C, pulse-type cluster; AMP, ampicillin; AMC, amoxicillin/clavulanate; CTX, cefotaxime; CAZ, ceftazidime; CVN, cefovecin; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; ETP, ertapenem; MEM, meropenem; TZP, piperacillin/tazobactam; ATM, aztreonam; NAL, nalidixic acid; ENR, enrofloxacin; CIP, ciprofloxacin; NOR, norfloxacin; LVX, levofloxacin; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; NET, netilmicin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; FFC, florfenicol; FOF, fosfomycin; CA-human, community-acquired UTI; HA-human, hospital-patient UTI.

Chapter 5

Sharing of *K. pneumoniae* and *P. mirabilis* between companion animals and humans

5.1 Evidence of sharing of *Klebsiella pneumoniae* strains between healthy companion animals and co-habiting humans.

Full paper published at the *Journal of Clinical Microbiology*

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*The author jointly performed case enrolement. The author conducted the sample processing, *K. pneumoniae* isolation and identification, DNA extraction, MLST and PFGE analysis. The author conducted 50% of PCR assays. The author performed data and statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on the co-authors and reviewers revisions.

Partial results were presented as,

One Oral communication at the international congress 28th ECVIM-CA, 2018, Rotterdam, the Netherlands;

Two Poster communications at the international congress 28th ECCMID, 2018, Madrid, Spain; and at the international congress CIISA Congress 2018, 2018, Lisbon, Portugal.

Evidence of *Klebsiella pneumoniae* sharing between healthy companion animals and co-habiting humans

Cátia Marques[a], Adriana Belas[a], Catarina Aboim[a], Patrícia Cavaco-Silva[b,c], Graça Trigueiro[d], Luís Telo Gama[a], Constança Pomba[a*]

[a]CIISA - Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal;
[b]Centro de Investigação Interdisciplinar Egas Moniz, Instituto Universitário Egas Moniz, Caparica, Portugal
[c]Technophage, Lisboa, Portugal
[d]Laboratório de Análises Clínicas Dr. Joaquim Chaves, Lisboa, Portugal

Abstract
This study aimed to characterise the faecal colonisation and sharing of *Klebsiella pneumoniae* strains between companion animals and humans living in close contact.
Faecal samples were collected from 50 healthy participants (24 humans, 18 dogs and 8 cats) belonging to 18 households. Samples were plated onto MacConkey agar plates (MCK) with and without cefotaxime or meropenem supplementation. Up to five *K. pneumoniae* colonies per participant were compared by pulsed-field gel electrophoresis (PFGE) after *Xba*I restriction. *K. pneumoniae* strains with unique pulse-types from each participant were characterised for antimicrobial susceptibility, virulence genes and multilocus sequence type (MLST). Faecal *K. pneumoniae* pulse-types were compared to those of clinical *K. pneumoniae* strains from animal and human patients with urinary tract infections (*n*= 104).
K. pneumoniae colonisation was detected in non-supplemented MCK in around 38% of dogs (*n*= 7) and humans (*n*= 9). *K. pneumoniae* isolated from dogs belonged to sequence type 17 (ST17), ST188, ST252, ST281, ST423, ST1093, ST1241, ST3398 and ST3399. None of the *K. pneumoniae* strains were multidrug-resistant or hypervirulent. Two households included multiple colonised participants. Notably, two colonised dogs within-household 15 (H15) shared a strain each (ST252 and ST1241) with one co-living human. One dog from H16 shared one PFGE undistinguishable *K. pneumoniae* ST17 strain with two humans from different households; however, the antimicrobial susceptibility phenotypes of these three strains differed. Two main virulence genotypes were detected, namely *fimH-1*/*mrkD*/*ycfM*/*entB*/*kfu* and *fimH-1*/*mrkD*/*ycfM*/*entB*/*kpn*.
These results highlight the potential role of dogs as reservoir of *K. pneumoniae* to humans and vice versa. Furthermore, to our best knowledge, this is the first report of healthy humans and dogs sharing *K. pneumoniae* strains that were undistinguishable by PFGE/MLST.

Keywords: *Klebsiella pneumoniae*, clonal relatedness, animal-human sharing, companion animals, dog, humans

Introduction

Klebsiella pneumoniae is an important nosocomial agent that is known to spread easily (Martim et al., 2016; Martin & Bachman, 2018). *K. pneumoniae* can also cause community onset infections in companion animals and humans and is the second most common Enterobacteriaceae species causing urinary tract infections (UTI) in humans (Gupta et al., 1999; Ewers et al., 2014b; Marques et al., 2018b).

Extended spectrum beta-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae are frequently multidrug-resistant, which leads to important therapeutic limitations (Marques et al., 2018b; ECDC, 2017). ESBL/Carbapenemase-producing *K. pneumoniae* strains are frequently reported worldwide and their dissemination is of great importance (Ewers et al., 2014b; ECDC, 2017).

Companion animals may become infected with *K. pneumoniae* high-risk clonal lineages, such as ST15, which is frequently a CTX-M-15-producer (Ewers et al., 2014b; Harada et al., 2016; Maeyama et al., 2018; Marques et al., 2018c). However, little is known about the role of healthy dogs and cats as reservoirs of such clonal lineages.

Gut colonisation by *K. pneumoniae* is strongly linked to subsequent extraintestinal infections in hospitalised human patients (Martin et al., 2016). Moreover, Enterobacteriaceae species that cause UTI, such as *K. pneumoniae*, are frequently part of the host gut microbiota (Martin et al., 2016). The gut of companion animals may be colonised by high-risk clonal lineages of *Escherichia coli* (Johnson et al., 2016) and *Enterococcus faecium* (Damborg et al., 2009), thus potentially acting as reservoirs to humans. In humans, most studies on *K. pneumoniae* faecal colonisation are focussed on hospitalised and/or infected patients and therefore less information is available regarding healthy humans (Martin et al., 2016; Martin et al., 2018). Studies on the population structure of *K. pneumoniae* strains colonising healthy dogs and cats are also lacking.

The transmission of pathogenic bacteria from companion animals to humans has been a growing matter of concern (Pomba et al., 2017). The close contact between companion animals and humans in modern society leads to greater chances of interspecies transmission of bacteria (Pomba et al., 2017), including through faecal contamination. Previous studies focussed on *E. coli* transmission dynamics have reported that the index strains from humans or dogs with UTI are extensively shared with other human and dog household members (Johnson & Clabots, 2006; Johnson, Clabots & Kuskowski, 2008a). Sharing of *E. coli* between healthy humans and dogs living in close contact has also been described (Johnson, Owens, Gajewski & Clabots, 2008b; Stenske et al., 2009; Harada et al., 2012b; Naziri, Derakhshandeh, Firouzi, Motamedifar & Shojaee Tabrizi, 2016). To our knowledge, studies on animal-human sharing have not been conducted regarding *K. pneumoniae*. However, this information is crucial to a better understanding of the epidemiology of this important pathogen.

In the current study, several households composed of healthy companion animals (dogs and cats) and humans living in close contact were enrolled to evaluate the frequency of *K. pneumoniae* colonisation and animal-human sharing. Additionally, this study aimed to characterise the population structure, antimicrobial resistance and virulence of faecal *K. pneumoniae* to aid the understanding of the role of healthy companion animals as reservoirs to humans and vice versa. A special focus regarding UTI was given by comparing the faecal

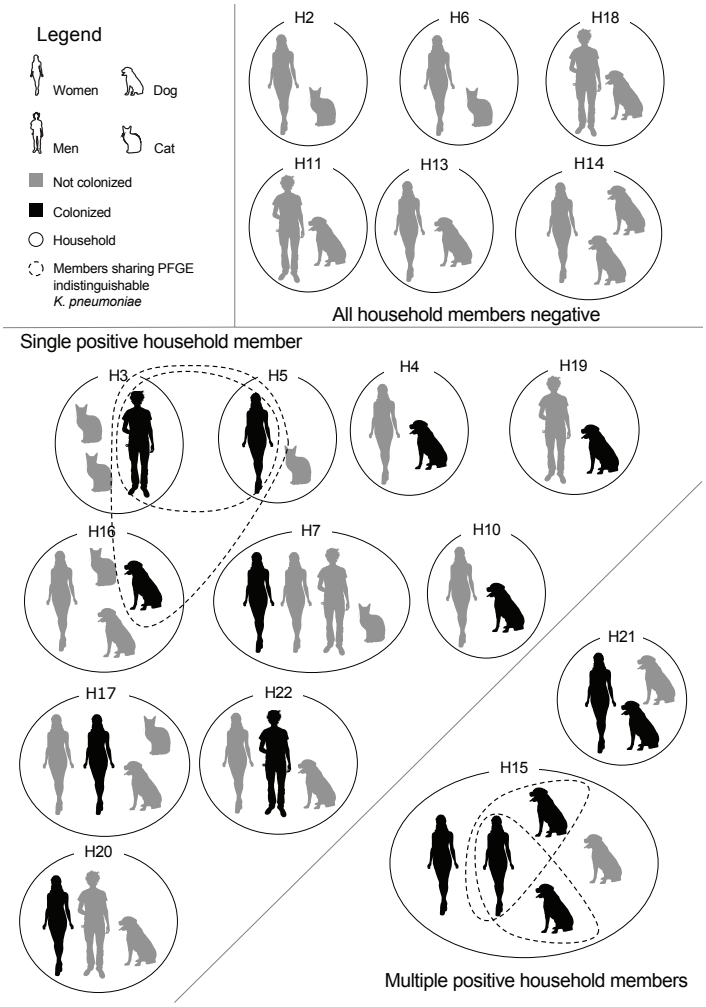
K. pneumoniae strains with a previously characterised collection of clinical *K. pneumoniae* isolates from humans, dogs and cats with UTI (Marques et al., 2018c).

Materials and methods

Study population

The study population included households with at least one human and one companion animal (dog or cat) living in close contact. Prior to inclusion, all human participants were informed of the main goals of the study and were asked to sign a consent form. Participants were considered "healthy" and were included in the study if no bacterial infection nor antimicrobial use was reported in the previous month. To ensure that inclusion was anonymous, households, humans and animals were coded with numbered letters H, Hu and A, respectively. A total of 50 participants (24 humans, 18 dogs and 8 cats) living in 18 households were enrolled in 2016. Therefore, the household composition varied in the number of humans and companion animals (Figure 1). Human participants had lived in the same household as the included companion animals for at least 6 months, except one cat that had been recently adopted to H16 (Figure 1).

Figure 1 - Faecal colonisation and sharing of *K. pneumoniae* among household members



All human participants were more than 18 years of age and 70.8% (*n*= 17/24) were women. Companion animal ages ranged from 2 months to 17 years, and 57.7% (*n*= 15/26) were females. Thirty-three percent (*n*= 8/24) of humans and 19.2% (*n*= 5/26) of companion animals had undergone antimicrobial treatment within the previous year. All cats lived exclusively indoors except one. All dogs had access to the outdoors, 83.3% (*n*= 15/18) lived indoors with the owners while, 16.7% (*n*= 3/18) stayed at private yards.

Sample collection and bacteriological methods

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. All faecal samples were collected using non-invasive methods after informed, written consent was obtained. Enrolled humans collected their own faecal samples and the faecal samples from the co-living companion animals into sterile containers. Immediately after collection, faecal samples were stored at 4ºC until processing.

One gramme of homogenised faecal sample was added to 10 mL of sterile 0.85% NaCl (Merk, Germany) solution and mixed thoroughly. Ten microlitres of faecal suspension were plated onto MacConkey agar plates (Scharlau, Spain) with or without 1.5 μg/mL of cefotaxime (Sigma-Aldrich, USA) or meropenem (Sigma-Aldrich, USA) supplementation. To improve detection of low number *K. pneumoniae*, one gramme of feces was added to 5 mL of sterile buffered peptone water (Biokar diagnostics, France), vortexed and incubated at 36±1ºC during 18h. A negative quality control consisting of buffered peptone water alone was also incubated. Following incubation, 1 μL of buffered peptone water faecal suspension was plated onto the MacConkey agar plates described above. MacConkey agar plates were incubated at 36±1ºC for 18h, followed by inspection for *K. pneumoniae* suspected colonies.

To guide presumptive *K. pneumoniae* identification, suspected colonies obtained from MacConkey agar plates were streaked onto UriSelect™ agar plates (Biorad, USA). Up to five *K. pneumoniae* suspected colonies per participant were isolated and stored in 20% glycerol (Sigma-Aldrich, USA) brain heart infusion broth (Biokar diagnostics, France) at -20 ºC until processing. Total DNA was extracted by boiling method and *K. pneumoniae* species confirmed by PCR as previously described (Féria et al., 2002; Padmavathy et al., 2012).

All faecal samples had a high number of colony forming units of Enterobacteriaceae after direct plating onto MacConkey agar plates, thus confirming sample viability.

K. pneumoniae population structure analysis

All *K. pneumoniae* isolates were compared by pulse field gel electrophoresis (PFGE) after 3h *Xba*I (New England Biolabs, USA) restriction. Restriction fragments were separated on a CHEF DR II-apparatus (Biorad, USA) using a 1% agarose gel (agarose pulse-field grade,

Nzytech - Genes and Enzymes, Portugal) and previously described electrophoresis conditions (5 to 20 s for 4 h followed by 25 to 50 s for 18 h at 14ºC, 6 V/cm^2) (Rodrigues et al., 2016).
K. pneumoniae strains with unique pulse-types from each animal or human were further typed by MLST according to the published consensus MLST scheme (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Collection of clinical UTI K. pneumoniae

The faecal *K. pneumoniae* PFGE restriction patterns from this study were compared with a previously typed collection of clinical *K. pneumoniae* strains isolated from dogs, cats (*n*= 27) and humans (*n*= 77 [*n*= 19 hospital patients and *n*= 58 community patients]) with UTI (Marques et al., 2018c).

Susceptibility testing

K. pneumoniae strains with unique pulse-types from each animal and human were tested for antimicrobial resistance to amoxicillin/clavulanate 30 μg, cefoxitin 30 μg, cefotaxime 30 μg, meropenem 10 μg, ciprofloxacin 5 μg, gentamicin 10 μg, amikacin 30 μg, nitrofurantoin 300 μg, chloramphenicol 30 μg, tetracycline 30 μg and trimethoprim/sulfamethoxazole 25 μg (Oxoid, Hampshire, UK). Antimicrobial susceptibility was conducted by disk diffusion according to CLSI guidelines (CLSI, 2017).

Antimicrobial resistance determinants

K. pneumoniae isolates that were not susceptible to at least one of the tested β-lactams, except ampicillin, were tested by PCR for the presence of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX\text{-}M\text{-}type}$, *bla*$_{CIT\text{-}type}$, *bla*$_{DHA\text{-}type}$, *bla*$_{MOX\text{-}type}$, *bla*$_{ACT\text{-}type}$, *bla*$_{FOX\text{-}type}$ and *bla*$_{MIR\text{-}type}$ β-lactamase genes using previously described primers (Pérez-Pérez & Hanson, 2002; Pomba et al., 2006; Guessennd et al., 2008).
K. pneumoniae strains with unique pulse-types were tested for the presence of efflux pump (*oqxAB*) (Kim et al., 2009c) and outer membrane proteins (ompK35 and ompK36) (Lee et al., 2007) coding genes.

Virulence genes

The *K. pneumoniae* strains that were tested for antimicrobial susceptibility were also screened by PCR for the presence of the following virulence genes: type-1 (*fimH-1*), type-3 (*mrkD*) and FimH-like (*kpn*) fimbriae adhesins (El Fertas-Aissani et al., 2013; Compain et al., 2014), outer membrane lipoprotein (*ycfM*) (El Fertas-Aissani et al., 2013), catecholate siderophore receptor (*iroN*) (Johnson et al., 2000), enterobactin (*entB*) (Compain et al., 2014), aerobactin (*iutA*) (Compain et al., 2014), iron transporter with phosphotransferase function (*kfu*) (Compain et al., 2014), yersiniabactin high-pathogenicity island ([YHPI], *irp-1*, *irp-2*, *fyuA*, *ybtS*) (Johnson & Stell, 2000; Schubert et al., 2000; Compain et al., 2014), serum resistance-associated outer

membrane lipoprotein (*traT*) (Johnson & Stell, 2000), regulator of mucoid phenotype A (*rmpA*) (Compain et al., 2014) and allantoin metabolism associated gene (*allS*) (Compain et al., 2014).

Data analysis

K. pneumoniae PFGE-patterns were compared using the Bionumerics software version 6.6, (BioMérieux, France) and Dice/unweighted pair group method with arithmetic mean (UMPGA) clustering method with a tolerance of 1.5% and a clustering cut-off of 80%. Previously proposed criteria for bacterial strain typing were used (Tenover et al., 1995).

The Fisher's exact test was used for comparisons between groups, with an alpha value of 0.05, using SAS statistical software package for Windows version 9.3 (SAS Institute Inc, Cary, USA).

K. pneumoniae STs from this study were compared with all known STs from the Institut Pasteur *K. pneumoniae* database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) through eBURST$_{V3}$ analysis (http://eburst.mlst.net/). *K. pneumoniae* STs were assigned to the same group if they shared identical alleles at 6 out of 7 loci with at least one other ST.

Results

K. pneumoniae colonisation was detected in 16 participants (7 dogs and 9 humans) from 12 households (Figure 1). All samples were negative for *K. pneumoniae* growth in MacConkey agar plates with cefotaxime or meropenem supplementation.

The faecal colonisation by *K. pneumoniae* was equally high in dogs (38.9%, *n*= 7/18) and in humans (37.5%, *n*= 9/24). However, *K. pneumoniae* colonisation was not detected in cats despite the use of pre-enrichment prior to plating.

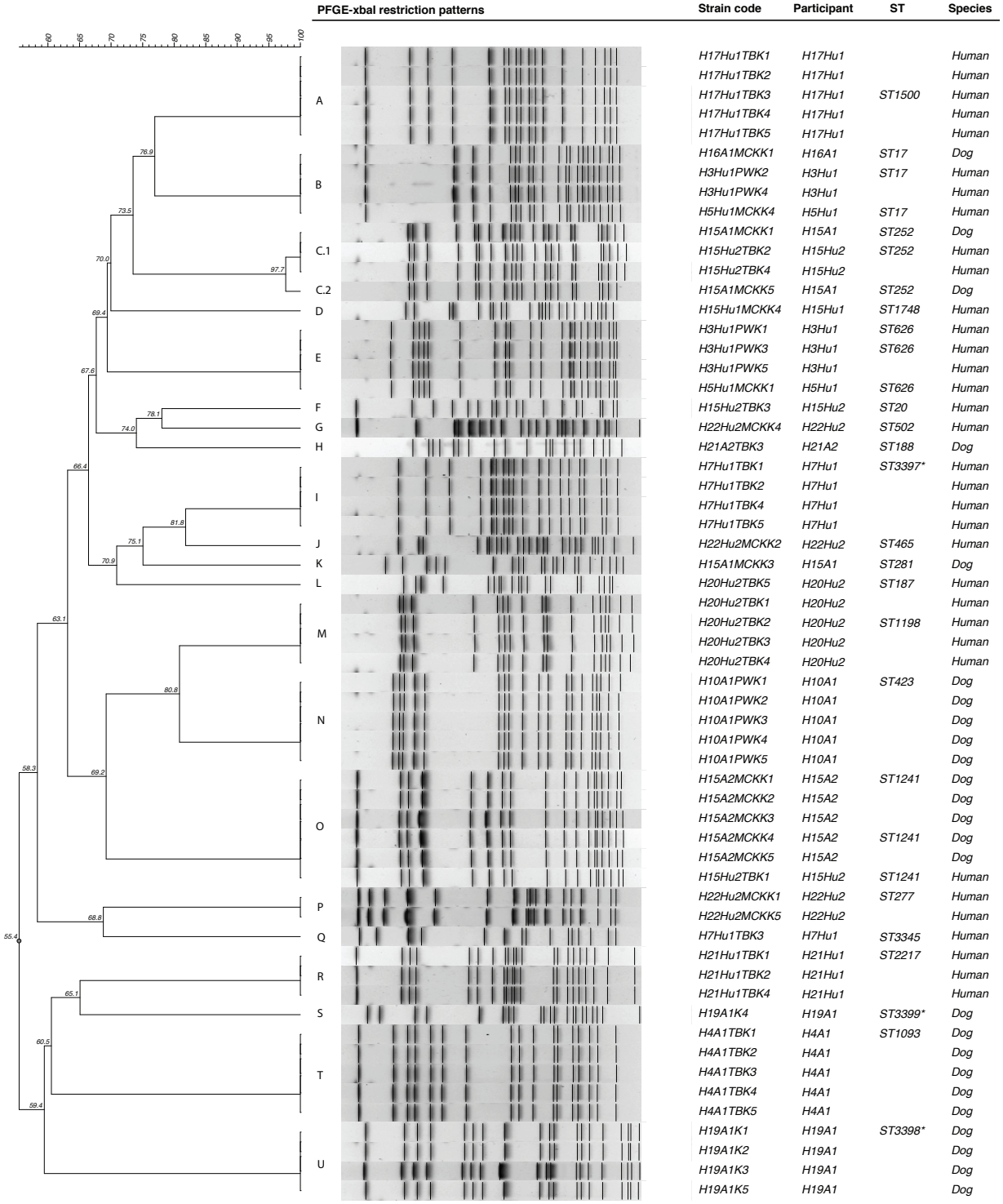
The majority of positive households (83.3%, *n*= 10/12) had a single colonised participant and therefore within-household sharing of *K. pneumoniae* was absent in these cases (Figure 1). The two households with multiple colonised participants included colonised humans and dogs (Figure 1).

A total of 59 *K. pneumoniae* isolates were obtained from the 16 positive participants and the number of isolates per participant ranged from one to five. The PFGE analysis revealed a total of 22 different restriction patterns (A to U) (Figure 2).

In samples from which two to five *K. pneumoniae* colonies were isolated, 61.5% (*n*= 8/13) of the participants were colonised by two or more different *K. pneumoniae* strains according to the PFGE results (Figure 2, Table 1). The PFGE analysis of the *K. pneumoniae* isolates from the participants living in household H21 showed that the human and dog were colonised by unrelated *K. pneumoniae* strains (pulse-type R and H, respectively) (Figure 2). In household H15, which was composed by two colonised humans and two colonised dogs, it is interesting to notice that while both humans were colonised by unrelated *K. pneumoniae* strains, the human H15Hu2 shared one *K. pneumoniae* strain undistinguishable by PFGE with dog-H15A1

(pulse-type C.1) and another with dog-H15A2 (pulse-type O) (Figure 2). In both dogs from household H15, the colonising *K. pneumoniae* strain was detected without pre-enrichment, while in the human H15Hu2 pre-enrichment was needed.

Figure 2- PFGE analysis of commensal *K. pneumoniae* from humans and animals living in close contact



Legend: *ST3397, ST3398 and ST3399 new STs described in this study.

Table 1 - Characterisation of faecal *K. pneumoniae* strains

Household	Household member	Strain ID	Pulse-type	Sequence type	Clonal complex[b]	Antimicrobial resistance[c]	Virulence
H3	Human Hu1	H3Hu1PWK1	E	ST626	-	None	*fimH-1, mrkD, ycfM, entB, kfu, allS*
H3	Human Hu1	H3Hu1PWK2	B	ST17	CC11	None	*fimH-1, mrkD, ycfM, entB, kpn*
H4	Dog A1	H4A1TBK1	T	ST1093	CC11	(AK), NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H5	Human Hu1	H5Hu1MCKK1	E	ST626	-	(NIT)	*fimH-1, mrkD, ycfM, entB, kfu, allS*
H5	Human Hu1	H5Hu1MCKK4	B	ST17	CC11	(CTX), (CN), (AK)	*fimH-1, mrkD, ycfM, entB, kpn*
H7	Human Hu1	H7Hu1TBK1	I	ST3397[a]	CC11	None	*fimH-1, mrkD, ycfM, entB, kfu, allS*
H7	Human Hu1	H7Hu1TBK3	Q	ST3345	CC11	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H10	Dog A1	H10A1PWK1	N	ST423	CC11	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H15	Dog A1	H15A1MCKK1	C.1	ST252	CC11	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H15	Dog A1	H15A1MCKK3	K	ST281	CC505	NIT	*fimH-1, mrkD, ycfM, entB, kpn, YHPI*
H15	Dog A1	H15A1MCKK5	C.2	ST252	CC11	(CN), (AK), NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H15	Dog A2	H15A2MCKK1	O	ST1241	-	NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H15	Human Hu1	H15Hu1MCKK4	D	ST1748	Singleton	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H15	Human Hu2	H15Hu2TBK1	O	ST1241	-	NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H15	Human Hu2	H15Hu2TBK2	C.1	ST252	CC11	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H15	Human Hu2	H15Hu2TBK3	F	ST20	CC11	CN, (AK), NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H16	Dog A1	H16A1MCKK1	B	ST17	CC11	(CN), (AK)	*fimH-1, mrkD, ycfM, entB, kpn*
H17	Human Hu1	H17Hu1TBK3	A	ST1500	CC11	NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H19	Dog A1	H19A1K1	U	ST3398[a]	Singleton	(CN), NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H19	Dog A1	H19A1K4	S	ST3399[a]	Singleton	(CN), NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H20	Human Hu2	H20Hu2TBK2	M	ST1198	CC11	C, NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H20	Human Hu2	H20Hu2TBK5	L	ST187	CC187	(CTX), NIT	*fimH-1, mrkD, ycfM, entB, kpn*

Table 1 (continuation)- Characterisation of faecal *K. pneumoniae* strains

Household	Household member	Strain ID	Pulse-type	Sequence type	Clonal complex[b]	Antimicrobial resistance[c]	Virulence
H21	Dog A2	H21A2TBK3	H	ST188	Singleton	(CN)	*fimH-1, mrkD, ycfM, entB, kfu*
H21	Human Hu1	H21Hu1TBK1	R	ST2217	Singleton	(CN), NIT	*fimH-1, mrkD, ycfM, entB, kfu*
H22	Human Hu2	H22Hu2MCKK1	P	ST277	CC11	(CN), NIT	*fimH-1, mrkD, ycfM, entB, kpn*
H22	Human Hu2	H22Hu2MCKK2	J	ST465	CC11	TE	*fimH-1, mrkD, ycfM, entB, kpn*
H22	Human Hu2	H22Hu2MCKK4	G	ST502	CC502	None	*fimH-1, mrkD, ycfM, entB, kpn*

Legend: *fimH-1*, type-1 adhesin; *mrkD*, type-3 adhesin; *kpn*, FimH-like adhesin; *ycfM*, outer membrane lipoprotein; *entB*, enterobactin; *kfu*, iron transporter with phosphotransferase function; YHPI, yersiniabactin high-pathogenicity island; *allS*, allantoin metabolism associated gene; AK, amikacin; C, chloramphenicol; CN, gentamicin; CTX, cefotaxime; NIT, nitrofurantoin; TE, tetracycline;

[a]ST3397, ST3398 and ST3399 new STs described in this study;

[b]Clonal complex (CC) were assigned based on the predicted founder ST based on a population snapshot by eBURST$_{V3}$ analysis of all *K. pneumoniae* Sequence types known until 31th of August 2018;

[c]intermediate resistance is indicated within parenthesis.

Two *K. pneumoniae* pulse-types, namely E and B, were shared between humans living in distinct households (Figure 1, 2). *K. pneumoniae* pulse-types E and B were shared by one human from household H3 and another from household H5. Furthermore, *K. pneumoniae* pulse-type B was also shared by the dog-H16A1 from household H16 (Figure 1, 2). The colonised human participants from household H3 and H5 and the human living in close contact (H16Hu1) with the colonised dog-H16A1 share the same workplace and thus are epidemiologically related. Notably, the colonised dog-H16A1 does not visit the workplace of human H16Hu1.

Overall, within-household human-animal sharing of *K. pneumoniae* strains occurred in 5.5% (*n*= 1/18) of all included households and in 8.3% (*n*= 1/12) of positive households. Considering the positive participants, there were a total of 20 human-animal pairs where potential within-household sharing was possible. Based on PFGE results, 10% (*n*= 2/20) of these human-animal potential pairs shared undistinguishable *K. pneumoniae* strains. Although several households included multiple human participants, none of the colonised humans shared *K. pneumoniae* strains with the co-living humans (*n*= 5 households) (Figure 1). The same was true for animals living with colonised dogs (*n*= 3 households) (Figure 1).

Overall, 27 *K. pneumoniae* strains were typed by MLST and characterised for antimicrobial resistance and for the presence of virulence genes.

A total of 21 STs, corresponding to the different PFGE pulse-types, were detected, thus revealing high *K. pneumoniae* population diversity in colonised dogs and humans (Table 1). Three novel *K. pneumoniae* STs were described, namely ST3397 to ST3399. In eBURSTv3 analysis, the novel ST3398 and ST3399 isolated from a dog were found to be singletons. The *K. pneumoniae* ST3397 isolated from a human was a double locus variant from ST65.

Most *K. pneumoniae* strains were susceptible to the tested antimicrobials. The only exception was the antimicrobial nitrofurantoin against which 70.4% (*n*= 19/27) of strains were not susceptible. Furthermore, several strains were intermediately resistant against gentamicin and/or amikacin (Table 1). The two *K. pneumoniae* strains with intermediate resistance to cefotaxime were negative for all the β-lactamase genes tested except for bla_{SHV}.

Two main virulence genotypes were detected, namely *fimH-1*/*mrkD*/*ycfM*/*entB*/*kfu* and *fimH-1*/*mrkD*/*ycfM*/*entB*/*kpn* (Table 1). The *allS* gene was only detected in *K. pneumoniae* strains from humans belonging to ST626 and to the novel ST3397. The yersiniabactin high-pathogenicity island was present in one strain from a dog belonging to ST281 (Table 1). All *K. pneumoniae* isolates were negative for *rpmA*, *iutA*, *iroN* and *traT* genes and positive for *ompK35* and *ompK36*. Moreover, only one strain lacked *oqxAB* genes by PCR.

The *K. pneumoniae* strains from the participants sharing identical pulse-type/STs also shared the same virulence genotype. However, the antimicrobial resistance phenotype was not always similar (pulse-type E and B) (Table 1).

The *K. pneumoniae* strains shared between the H15 human/dog pairs belonged to ST252 (pulse-type C.1) and ST1241 (pulse-type O) and also had identical antimicrobial resistance phenotype and an identical virulence genotype (Table 1). Both household H15 human participants reported that they allowed all the household dogs (*n*= 3) to lick their faces and sleep in their beds. No difference in dog-dog or dog-human behavior was noted regarding the three dogs living together. Interestingly, the dog-H15A1 was colonised by two variants (pulse-type C.1 and C.2) of the same *K. pneumoniae* pulse-type (Figure 1, Table 1). The *K. pneumoniae* strains (pulse-type B) shared between the two humans and one dog from different households all belonged to ST17, however, these strains presented different antimicrobial resistant phenotypes (Table 1).

Some of the faecal *K. pneumoniae* strains, mostly from humans, showed a ≥80% similarity to strains from humans with UTI (Figure 3). Of note, dog H10A1 was colonised with a *K. pneumoniae* strain that was closely related (92.3% similarity) to a clinical ST423 CTX-M-15-producing *K. pneumoniae* strain (PC25/15B) from a human (Figure 3). The dog and human from household H10 did not have prior clinical history of UTI, were not under antimicrobial treatment in the last year, and had no contact with the hospital environment.

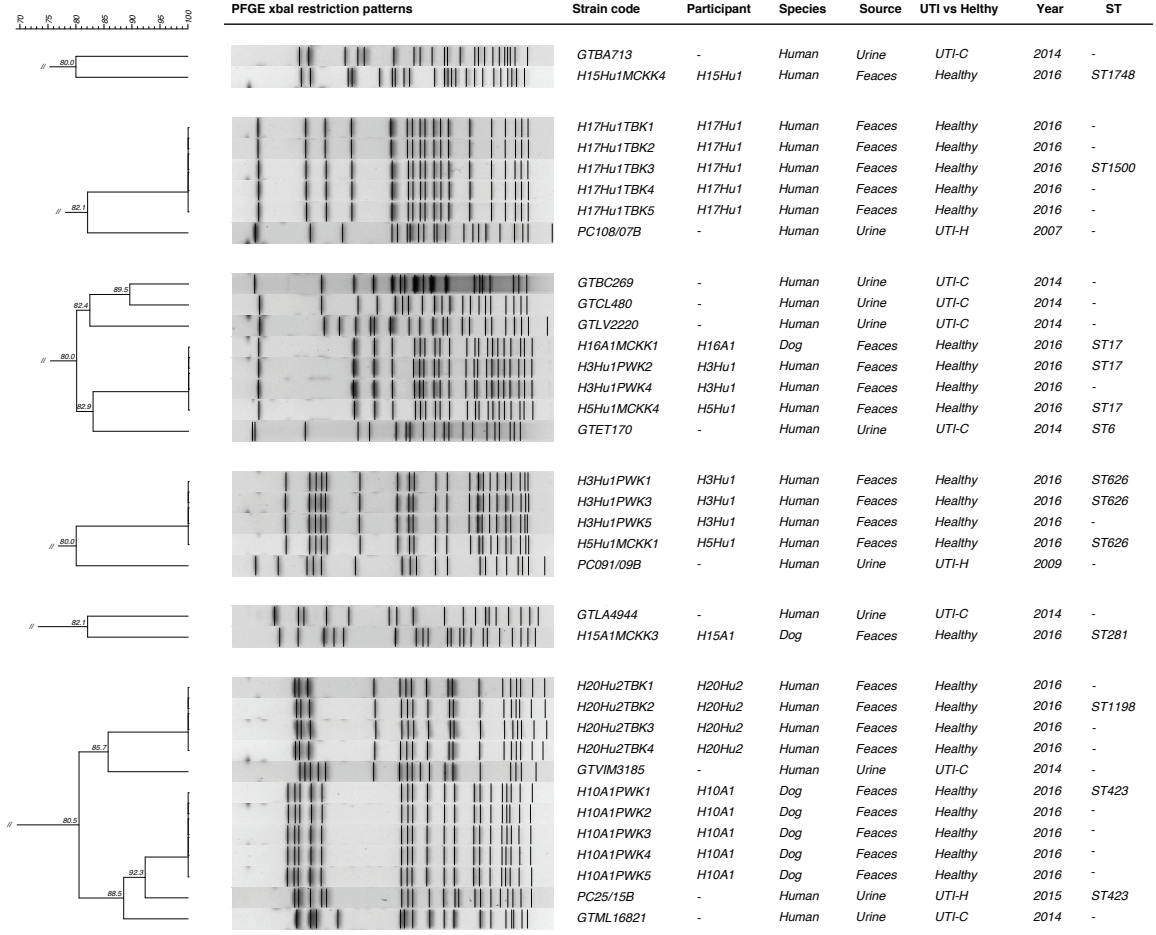
Discussion

To our best knowledge, this is the first report of the faecal colonisation and sharing of *K. pneumoniae* clonal lineages between healthy humans and dogs living in close contact.

The high *K. pneumoniae* population diversity detected in this study is in line with that in previous reports (Harada et al., 2016; Maeyama et al., 2018; Holt et al., 2015). There are several *K. pneumoniae* STs disseminated worldwide, including in Portugal, that are considered to be high-risk clonal lineages or are recognised as important international outbreaks clones (Manageiro et al., 2015; Navon-Venezia et al., 2017). Considering that the gut is a reservoir of pathogenic Enterobacteriaceae (Martin et al., 2016), it is interesting to notice that only the high-risk ST17 and the international outbreak ST20 *K. pneumoniae* clonal lineages were detected in this study. The *K. pneumoniae* high-risk clonal lineages are frequently ESBL and carbapenemase-producers (Navon-Venezia et al., 2017). The fact that most of the faecal *K. pneumoniae* strains from this study were susceptible to β-lactams may explain the low frequency of high-risk clonal lineages detected.

K. pneumoniae ST15, which is a high-risk clonal lineage, seems to predominate among clinical CTX-M-15-producing strains from companion animals from several countries (Ewers et al., 2014b; Harada et al., 2016; Maeyama et al., 2018; Marques et al., 2018c). In a previous study conducted in dogs and cats with UTI from Portugal, most uropathogenic *K. pneumoniae* isolates also belonged to ST15 (Marques et al., 2018c). The absence of colonised dogs with *K. pneumoniae* ST15 was therefore a surprise.

Figure 3 - Clusters of *K. pneumoniae* isolated from clinical UTIs and from faecal samples of human and companion animal origin



PFGE xbal restriction patterns	Strain code	Participant	Species	Source	UTI vs Helthy	Year	ST
	GTBA713	-	Human	Urine	UTI-C	2014	-
	H15Hu1MCKK4	H15Hu1	Human	Feaces	Healthy	2016	ST1748
	H17Hu1TBK1	H17Hu1	Human	Feaces	Healthy	2016	-
	H17Hu1TBK2	H17Hu1	Human	Feaces	Healthy	2016	-
	H17Hu1TBK3	H17Hu1	Human	Feaces	Healthy	2016	ST1500
	H17Hu1TBK4	H17Hu1	Human	Feaces	Healthy	2016	-
	H17Hu1TBK5	H17Hu1	Human	Feaces	Healthy	2016	-
	PC108/07B	-	Human	Urine	UTI-H	2007	-
	GTBC269	-	Human	Urine	UTI-C	2014	-
	GTCL480	-	Human	Urine	UTI-C	2014	-
	GTLV2220	-	Human	Urine	UTI-C	2014	-
	H16A1MCKK1	H16A1	Dog	Feaces	Healthy	2016	ST17
	H3Hu1PWK2	H3Hu1	Human	Feaces	Healthy	2016	ST17
	H3Hu1PWK4	H3Hu1	Human	Feaces	Healthy	2016	-
	H5Hu1MCKK4	H5Hu1	Human	Feaces	Healthy	2016	ST17
	GTET170	-	Human	Urine	UTI-C	2014	ST6
	H3Hu1PWK1	H3Hu1	Human	Feaces	Healthy	2016	ST626
	H3Hu1PWK3	H3Hu1	Human	Feaces	Healthy	2016	ST626
	H3Hu1PWK5	H3Hu1	Human	Feaces	Healthy	2016	-
	H5Hu1MCKK1	H5Hu1	Human	Feaces	Healthy	2016	ST626
	PC091/09B	-	Human	Urine	UTI-H	2009	-
	GTLA4944	-	Human	Urine	UTI-C	2014	-
	H15A1MCKK3	H15A1	Dog	Feaces	Healthy	2016	ST281
	H20Hu2TBK1	H20Hu2	Human	Feaces	Healthy	2016	-
	H20Hu2TBK2	H20Hu2	Human	Feaces	Healthy	2016	ST1198
	H20Hu2TBK3	H20Hu2	Human	Feaces	Healthy	2016	-
	H20Hu2TBK4	H20Hu2	Human	Feaces	Healthy	2016	-
	GTVIM3185	-	Human	Urine	UTI-C	2014	-
	H10A1PWK1	H10A1	Dog	Feaces	Healthy	2016	ST423
	H10A1PWK2	H10A1	Dog	Feaces	Healthy	2016	-
	H10A1PWK3	H10A1	Dog	Feaces	Healthy	2016	-
	H10A1PWK4	H10A1	Dog	Feaces	Healthy	2016	-
	H10A1PWK5	H10A1	Dog	Feaces	Healthy	2016	-
	PC25/15B	-	Human	Urine	UTI-H	2015	ST423
	GTML16821	-	Human	Urine	UTI-C	2014	-

Legend: UTI-C, community patient with UTI; UTI-H, hospitalised patient with UTI.

Several studies have reported the colonisation and sharing of *E. coli* strains between companion animals and humans (Johnson & Clabots, 2006; Johnson et al., 2008a; Johnson et al., 2008b; Stenskeet al., 2009; Harada et al., 2012b; Naziri et al., 2016). To our best knowledge, data on *K. pneumoniae* is still lacking. The use of PFGE over whole genome sequencing (WGS) could be considered a limitation of the current study since the latter is more discriminatory and necessary to definitely ascertain the similarity of bacterial strains. However, previous studies on *K. pneumoniae* outbreaks have found that WGS based phylogeny is consistent with the PFGE and MLST data combined, especially in strains differing in less than 3 bands (Marsh et al., 2015; Sabirova et al., 2016). Therefore, the detection of dogs and a human living in the same household (H15) colonised by *K. pneumoniae* strains belonging to the same ST, with undistinguishable PFGE restriction patterns, with an identical antimicrobial resistance phenotype and an identical virulence genotype is strongly suggestive of human-dog *K. pneumoniae* sharing.

Household H15 is also remarkable because although 2 humans and 2 dogs were colonised by *K. pneumoniae*, within-household sharing was only detected between human-dog pairs. The absence of human-human or dog-dog sharing in this study is likely related to the low number

of households with multiple colonised humans or dogs. The *K. pneumoniae* ST252 clonal lineage shared between the human H15Hu2 and dog H15A1 has been previously detected in faecal samples from hospitalised patients and long-term care facility residents from Portugal (Papagiannitsis et al., 2015; Rodrigues et al., 2017). This could suggest that colonisation had human origin. However, the higher *K. pneumoniae* ST252 and ST1241 faecal burden detected in these dogs could point to dog-to-human transmission. Allowing the dog to lick the face has been suggested as a risk factor for dog-human *E. coli* sharing (Naziri et al., 2016). However, in household H15 this was not a determining factor. A common source of *K. pneumoniae* acquisition should also be hypothesised, but since the three dogs had a common living environment and behaviors, it would be likely that the three dogs would be colonised by the same strains. *K. pneumoniae* is known to spread easily (Martin et al., 2016), therefore, additional studies are necessary to clarify its routes of human-dog dissemination.

The two humans and one dog living in different households that were colonised by PFGE undistinguishable *K. pneumoniae* ST17 strains were epidemiologically related and therefore the *K. pneumoniae* transmission could have occurred through direct (human-human) or indirect (human-dog) contact. However, it should be noted that the antimicrobial resistance phenotypes of these *K. pneumoniae* ST17 strains differed. Thus, since the *K. pneumoniae* ST17 clonal lineage is disseminated worldwide (Navon-Venezia et al., 2017), the circulation of this strain in the community and the colonisation of these participants through unrelated sources is the likely explanation. For instance, retail meat has been pointed to as a potential source of uropathogenic *K. pneumoniae* to humans (Davis et al., 2015). The detection of a high-risk *K. pneumoniae* clonal lineage colonising a healthy dog highlights its possible role as a reservoir. Furthermore, other *K. pneumoniae* clonal lineages that were detected in dogs, namely ST188, ST252, ST281, ST423 and ST1093, have also been previously implicated in human infections (Davis et al., 2015; Ito et al., 2015; Papagiannitsis et al., 2015; da Silva et al., 2018; Garza-Ramos et al., 2018).

The colonisation of humans and dogs by *K. pneumoniae* was equally high (~38%) and 10% of the potential within-household human-animal pairs shared *K. pneumoniae* strains undistinguishable by PFGE. Since this study relied on standard culture procedures, *K. pneumoniae* colonisation and sharing could be underestimated due to the overgrowth of other Enterobacteriaceae species. Nevertheless, a previous study from India has reported that only 26% of the healthy dogs were colonised by *K. pneumoniae* (Sharif et al., 2017). Considering that 61.5% of participants were colonised by multiple strains, it can be speculated that the PFGE typing of a higher number of colonies per sample could be advantageous in future studies to detect additional sharing pairs. The nasopharynx is also a *K. pneumoniae* colonisation site (Martin et al., 2018). In retrospect, we find that the study of nasopharyngeal colonisation could have undisclosed further epidemiological links between colonised dogs and humans. The absence of colonised cats in this study may be related to the number of cats

tested, since infections caused by *K. pneumoniae* have been previously reported in cats (Marques et al., 2018c).

The frequency of *K. pneumoniae* virulence genes agrees with previously published data (El Fertas-Aissani et al., 2013; Compain et al., 2014; Holt et al., 2015). The absence or low frequency of virulence genes associated with higher *K. pneumoniae* invasiveness (Holt et al., 2015) is a positive outcome from this study. The first hypervirulent *K. pneumoniae* ST23 detected in Portugal was only recently described in a human patient (Pereira et al., 2017). Therefore, the absence of hypervirulent *K. pneumoniae* clonal lineages in this study was expected.

According to the annual report of the European Antimicrobial Resistance Surveillance Network, Portugal is among the countries with a higher frequency of resistance to third-generation cephalosporins, carbapenems and fluoroquinolones in invasive *K. pneumoniae* strains (ECDC, 2017). Additionally, a high frequency of faecal colonisation by ESBL/AmpC-producing *K. pneumoniae* has been reported in long-term care facility residents from Portugal (Rodrigues et al., 2017). The high susceptibility and lack of multidrug-resistant *K. pneumoniae* isolates in the present study is, therefore, considered a positive finding. Nevertheless, these results may be a consequence of the study design due to the limited sample size and because it relied on healthy humans and animals without reported infections or antimicrobial use in the prior month.

Another interesting finding from this study was the detection of one healthy dog (H10A1) colonised by a *K. pneumoniae* ST423 strain that was 92.3% similar by PFGE to one strain isolated from an unrelated hospitalised patient with UTI. The use of WGS is warranted to fully disclose the relatedness of these strains; nevertheless, this finding should not be neglected. *K. pneumoniae* can cause other important infections, such as pneumonia (Martin et al., 2016; Holt et al., 2015). For this reason, future comparative studies should include *K. pneumoniae* strains from other clinical origins to further understand the role of dogs as reservoirs of pathogenic *K. pneumoniae*. In fact, the *K. pneumoniae* clonal lineages detected in dogs from this study have been previously isolated from several types of human infections besides UTI (Papagiannitsis et al., 2015; Ito et al., 2015; da Silva et al., 2018; Garza-Ramos et al., 2018).

To conclude, this study presents novel epidemiological data regarding *K. pneumoniae* colonisation and suggests that healthy humans and dogs may share similar *K. pneumoniae* clonal lineages. The role of dogs as reservoirs of *K. pneumoniae* clonal lineages previously described in human infections is noteworthy, even though those strains were neither multidrug resistant nor hypervirulent. Some questions remain to be answered regarding the routes of transmission and persistence of *K. pneumoniae* colonisation over time in co-living humans and dogs. Future studies using longitudinal designs should be conducted to clarify these issues regarding infected and healthy companion animals. Meanwhile, good hygiene practices and proper faecal disposal should be advised to dog caretakers to minimise the chances of direct or indirect *K. pneumoniae* interspecies transmission.

5.2 Human and companion animal interspecies sharing of faecal *Proteus mirabilis*.

Paper in preparation for submission**

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*The author jointly performed case enrolement. The author conducted the sample processing, *P. mirabilis* isolation and identification, DNA extraction, PCR assays, PFGE analysis and performed data and statistical analysis. The author wrote the initial draft of the manuscript and improved the manuscript based on the supervisor revisions.

**Due to technical issues, arround 25% of *P. mirabilis* strains could not be typed by PFGE until this thesis submission. The manuscript here presented was written considering available data at this time.

Partial results were presented as,

One Poster communication at the international congress 28th ECCMID, 2018, Madrid, Spain.

Human and companion animal interspecies sharing of faecal *Proteus mirabilis*.

Cátia Marques[a], Adriana Belas[a], Patrícia Cavaco-Silva[b,c], Graça Trigueiro[d], Luís Telo Gama[a], Constança Pomba[a*]

[a]CIISA - Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal;
[b]Centro de Investigação Interdisciplinar Egas Moniz, Instituto Universitário Egas Moniz, Caparica, Portugal
[c]Technophage, Lisboa, Portugal
[d]Laboratório de Análises Clínicas Dr. Joaquim Chaves, Lisboa, Portugal

Abstract
This study aimed to determine the colonisation and sharing of *P. mirabilis* strains between healthy companion animals and humans living in close contact. It also aimed to compare the population structure of faecal and uropathogenic *P. mirabilis* strains from human and companion animal origin.
Eighteen households (24 humans, 18 dogs, 8 cats) with at least one human and one animal were studied. Faecal samples were plated onto MacConkey and Hektoen agar plates with and without pre-enrichment. *P. mirabilis* PFGE analysis (*Not*I; Dice/UPGMA; 1.5% tolerance) was conducted in households with multiple positive participants (up to 5 strains/participant) and in 70% of the remaining households. Antimicrobial susceptibility testing and screening of virulence genes was performed. Faecal *P. mirabilis* pulse-types were compared with uropathogenic clinical strains from human and companion animal origin (*n*= 183).
Forty-Nine *P. mirabilis* strains belonging to three humans (12.5%, *n*= 3/24) and eight dogs (44.4%, *n*= 8/18) were isolated from eight households (44.4%, *n*= 8/18). Dog colonisation was significantly higher (*P* = 0.0329). Three households had multiple colonised participants (dog/dog, *n*= 1; human/dog, *n*= 2). One human/dog and the dog/dog pairs shared closely related *P. mirabilis* strains. Some human and dog *P. mirabilis* faecal strains clustered with clinical *P. mirabilis* strains (>80% Dice/UPGMA index). Strains were susceptible to most antimicrobials and frequently encoded *HmpA*/*HmpB, mrpA*, *pmpA* and *ucaA*.
To our knowledge, this is the first report of dogs and humans living in close contact sharing closely related *P. mirabilis* strains. The high frequency of colonisation in dogs and inter/intra-species sharing suggests the role of dogs as reservoirs of *P. mirabilis* to humans and dogs.

Keywords: *Proteus mirabilis*, Dogs, Humans, Faeces, Sharing

Introduction

Proteus mirabilis is a common cause of urinary tract infections (UTI) (Drzewiecka, 2016). In humans, *P. mirabilis* is frequently associated with complicated, recurrent and/or catheter associated UTIs (CAUTI) (Drzewiecka, 2016; Armbruster et al., 2018). UTIs caused by *P. mirabilis* are medically demanding because they are prone to persistence and to secondary complications such as urolithiasis, obstruction and pyelonephritis (Armbruster et al., 2018). *P. mirabilis* is also relevant in companion animals with UTI since it is the second most common Gram-negative bacteria isolated from dogs and cats (Marques et al., 2018b).

The increasing number of companion animals in modern society has raised concerns regarding the role of dogs and cats as reservoirs of pathogenic and/or antimicrobial resistant bacteria (Damborg et al., 2016; Pomba et al., 2017). In a recent study using PFGE, a high number of clusters (cluster >80% Dice/UPGMA index) were shown to contain clinical *P. mirabilis* strains isolated from humans and companion animals (dogs and cats) with UTI (Marques et al., 2018a).

The closer contact between owners and their pets increases the opportunities for pathogen interchange through direct and indirect contact (Damborg et al., 2016; Pomba et al., 2017). In fact, healthy dogs may share colonising *E. coli* strains with humans living in the same household (Johnson et al., 2008b; Stenske et al., 2009; Harada et al., 2012b; Naziri et al., 2016). The gut is pointed as the major reservoir of uropathogenic bacteria, including *P. mirabilis* (Drzewiecka, 2016). Notably, healthy dogs have been found to become colonised by *E. coli* clonal lineages known to cause UTI in humans, namely ST131 (Johnson et al., 2016). The worldwide *P. mirabilis* clonal epidemiology is still poorly understood due, to some extent, to the lack of portable *P. mirabilis* molecular typing methods such as MLST. Few studies have addressed the gut *P. mirabilis* colonisation of healthy dogs and cats (Balish et al., 1977; Gaastra et al., 1996). Furthermore, to our best knowledge, there is a lack of studies characterising the *P. mirabilis* sharing between companion animals and humans living in close contact.

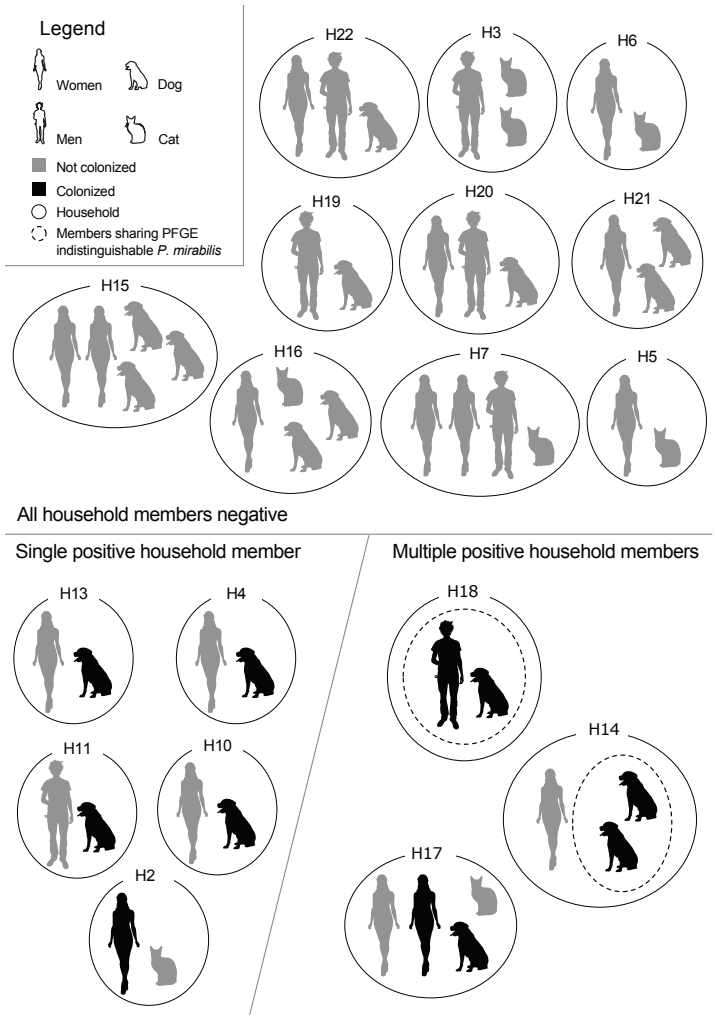
This study aimed to evaluate the *P. mirabilis* faecal colonisation and sharing between companion animals and humans living in the same household. Furthermore, the faecal *P. mirabilis* strains where compared by PFGE with clinical strains from companion animals and humans with UTI.

Materials and methods

Study population

Households composed of healthy humans and companion animals (dogs and/or cats) were enrolled in 2016. The health status of participants was accessed through a questionnaire and “healthy” was defined as the absence of known bacterial infections and/or antimicrobial use in the prior month. Human participants were informed of the main goals of the study and were required to sign a consent form. The studied population, included a total of 50 individuals (24 humans, 18 dogs and 8 cats) comprising 18 households. All human participants had lived together with the corresponding companion animals for at least 6 months prior to the study, except one cat that had been recently adopted to H16. Each family was composed of at least one human and one companion animal (dog or cat), yet the number of humans and companion animals varied among households (Figure 1). Enrolment was anonymous, therefore households, humans and animals were numbered and coded with the letters H, Hu and A, respectively.

Figure 1 - Faecal colonisation and within-household sharing of *P. mirabilis*



Sample collection and bacteriological methods

Ethical approval was obtained from the Comissão de Ética e Bem-Estar Animal (CEBEA) from the Faculty of Veterinary Medicine of the University of Lisbon. Human participants were asked to collect their own faecal samples, as well as the faecal samples from their pets, into sterile containers using non-invasive methods. Faecal samples were immediately stored at 4ºC until processing.

After sample homogenisation, one gramme of faeces was added to 10 mL of sterile 0.85% NaCl (Merk, Germany) solution and vortexed. Following thorough mixing, 10 μL of faecal suspension were plated onto MacConkey agar plates (Scharlau, Spain) and Hektoen Enteric agar plates (Biokar diagnostics, France). Ten microlitres of faecal suspension were also plated onto MacConkey agar plates supplemented with 1.5 μg/mL of cefotaxime (Sigma-Aldrich, USA) or meropenem (Sigma-Aldrich, USA).

One gramme of faeces was added to 5 mL of tetrathionate broth (tetrathionate broth base [Oxoid, UK] supplemented with iodine [AlliedSignal, Germany] and potassium iodide [Scharlau, Spain] according to the manufacturer instructions) to improve detection of *P. mirabilis*. The faecal suspention in tetrathionate broth was homogenised and incubated at

36±1ºC during 18h prior to plating in the agar plates described above. One tube of tetrathionate broth without faecal sample and one tube of tetrathionate broth inoculated with known *P. mirabilis* strains were included as negative and positive quality controls, respectively.
After incubation at 36±1ºC during 18h, all agar plates were inspected for the presence of *Proteus* spp. suspected colonies. When present, up to five colonies of each participant were isolated and stored in 20% glycerol (Sigma-Aldrich, USA) brain heart infusion broth (Biokar diagnostics, France) at -20 ºC until further analysis. Growth of a high number of colony forming units of Enterobacteriaceae after direct plating in MacConkey agar plates were deemed warrant as a quality control of sample viability.

Bacteria identification

Total DNA extraction of each suspected isolate of *Proteus* spp. was processed by boiling method (Féria et al., 2002) and species identification was conducted by PCR using previously described species-specific primers (Stankowska et al., 2008).

Population structure analysis

The clonal relatedness of all *P. mirabilis* isolates obtained from households with multiple colonised members was determined by pulse field gel electrophoresis (PFGE) after overnight *Not*I (New England Biolabs, USA) restriction. *P. mirabilis* from 70% of households with a single colonised member were also characterised. Restriction fragments were resolved in a 1% agarose gel (Agarose pulse-field grade, Nzytech - Genes and Enzymes, Portugal) using previously described electrophoresis conditions (1-30 s for 8 h followed by 30-70 s for 16 h at 14ºC, 6 V/cm^2) on a CHEF DR II-apparatus (Biorad, USA) (Sabbuba et al., 2003).

Clinical UTI P. mirabilis collection

The PFGE restriction patterns of faecal *P. mirabilis* strains from healthy companion animals and humans were compared to a previously characterised collection of clinical *P. mirabilis* isolated from companion animals (*n*= 107) and humans (*n*= 76 [*n*= 26 hospital patients and *n*= 50 community patients]) with UTI (Marques et al., 2018a).

Susceptibility testing

All unique *P. mirabilis* pulse-type/participant strains and one isolate per participant from untyped households were tested for antimicrobial resistance by disk diffusion. Susceptibility testing was conducted according to CLSI guidelines (CLSI, 2017; CLSI, 2018) for the following antimicrobials: ampicillin 10 μg, amoxicillin/clavulanate 30 μg, cefoxitin 30 μg, cefotaxime 30 μg, meropenem 10 μg, ciprofloxacin 5 μg, gentamicin 10 μg, amikacin 30 μg, fosfomycin 50 μg, tetracycline 30 μg, choramphenicol 30 μg and trimethoprim/sulfamethoxazole 25 μg (Oxoid, Hampshire, UK).

Human CLSI breakpoints (CLSI, 2017) were used since Veterinary CLSI breakpoints (CLSI, 2018) are either not determined for the tested antimicrobials or are the same as recommended in the Human CLSI guidelines. The only exception is the gentamicin breakpoint that varies by 1mm in the intermedium/susceptible categorisation (CLSI, 2018; CLSI, 2017).

Virulence genes

All *P. mirabilis* tested for antimicrobial resistance were also screened for the presence of the urothelial cell adhesion fimbriae (UCA/NAF [*ucaA*]), the mannose-resistant *Proteus*-like fimbriae (MR/P [*mrpA*]), the *Proteus mirabilis* fimbriae (PMF [*pmfA*]) and HpmA/HpmB haemolysin (*HpmA* and *HpmB*) codifying genes by PCR using previously described primers (Sosa et al., 2006; Cestari et al., 2013).

Data analysis

The Fisher exact test was used to compare groups using the SAS statistical software package for Windows, version 9.3 (SAS Institute Inc, Cary, North Carolina, USA).

The Bionumerics software version 6.6 (BioMérieux, Marcy-l'Étoile, France) was used to compare the *P. mirabilis* restriction PFGE-patterns using Dice/UPGMA clustering method with a tolerance of 1.5% and a clustering cut-off of 80%.

Results

All human participants had more than 18 years of age. Women were over represented (70.8%, *n*= 17/24) and 33.3% (*n*= 8/24) of human participants underwent antimicrobial treatment in the prior year. Companion animals had ages ranging from 2 months to 17 years old, 19.2% (*n*= 5/26) underwent antimicrobial treatment in the prior year and 57.7% (*n*= 15/26) were females. Only one cat had access to the outdoors, 83.3% (*n*= 15/18) of dogs lived indoors while the remaining dogs lived in private yards.

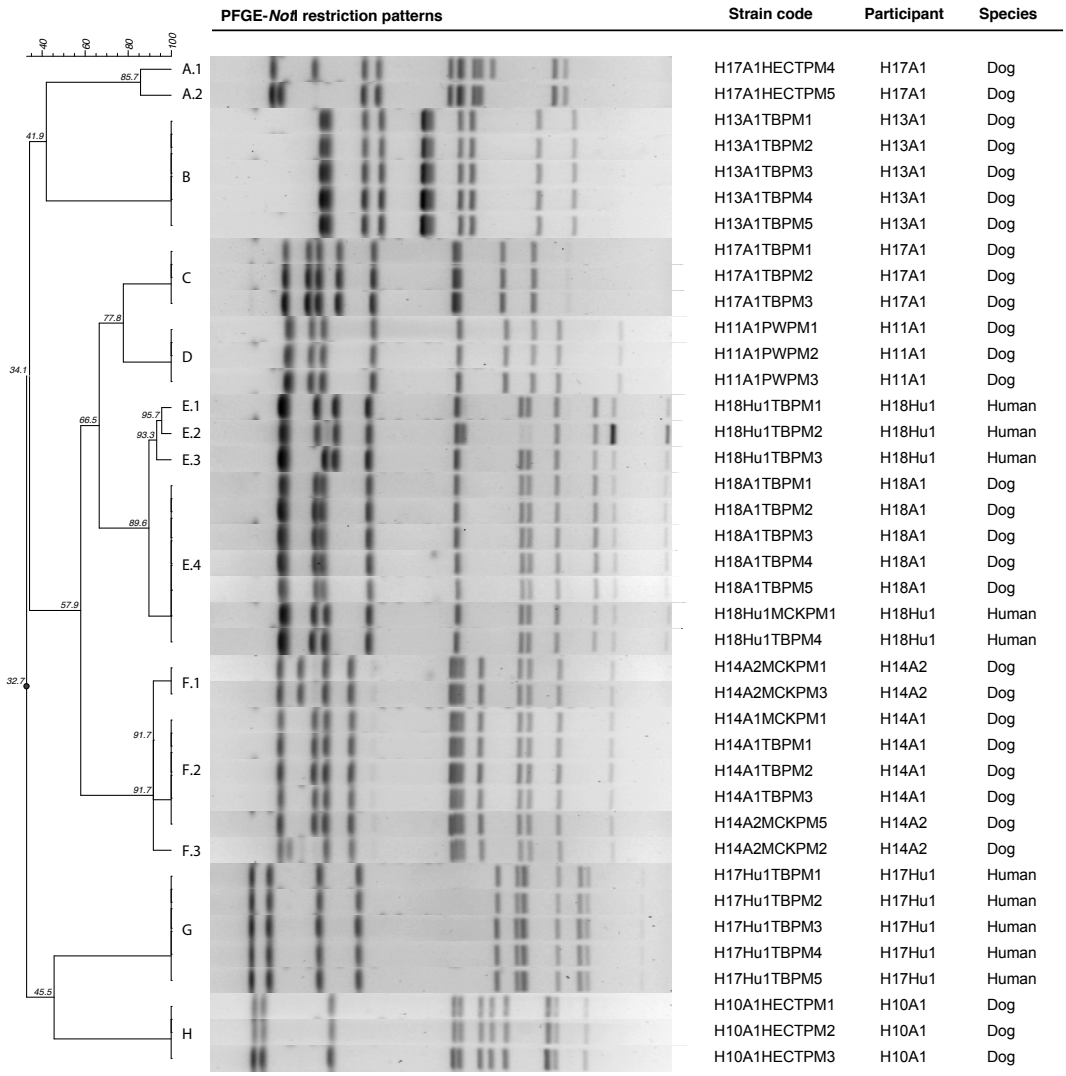
A total of 11 participants from 8 households had faecal colonisation by *P. mirabilis* (Figure 1). The use of tetrathionate broth was necessary for the detection of *P. mirabilis* in 36% (*n*= 4/11) of faecal samples of human and animal origin. Nevertheless, all the faecal samples (*n*= 50) were negative for *P. mirabilis* growth in cefotaxime and meropenem supplemented agar plates. When considering each participant species by itself, the *P. mirabilis* faecal colonisation was significantly higher (*P* = 0.0329) in dogs (44.4%, *n*= 8/18) than in humans (12.5%, *n*= 3/24). None of the cats had detectable *P. mirabilis* in faecal samples despite the use of tetrathionate broth prior to plating.

Most positive households included at least one colonised dog (87.5%, *n*= 7/8) (Figure 1). Interestingly, the only household containing solely a colonised human (H2), was composed of a non-colonised cat. Five out of the 8 positive households had a single participant colonised and therefore within-household sharing of *P. mirabilis* between these human/animal pairs was

absent (Figure 1). Two of the three households with multiple colonised participants included colonised human/dog pairs (H17, H18), while the third household (H14) had two positive dogs (Figure 1).

PFGE analysis was conducted in 39 *P. mirabilis* isolated from 3 households with multiple colonised participants (*n*= 28) and from 3 households with single colonised participants (*n*= 11). A total of 8 *P. mirabilis* PFGE clusters (from A to H) were identified. None of the *P. mirabilis* from different households clustered together, therefore *P. mirabilis* was not shared across-households (Figure 2).

Figure 2- PFGE analysis of *P. mirabilis* colonising the gut of humans and dogs



The PFGE analysis of the multiple *P. mirabilis* isolates obtained from each participant revealed that two dogs (H14A2 and H17A1) and one human (H18Hu1) had several variants of the same colonising strain (Figure 2, Table 1). Following Tenover et al. (1995) criteria these variants are consistent with single genetic events and therefore these strains are likelly closely related.

Table 1- Characterisation of faecal *P. mirabilis* strains

Household	Household member	Strain ID	Pulse-type	Antimicrobial resistance	Virulence
H2	Human Hu1	H2Hu1MCKPM1	Not determined	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H4	Dog A1	H4A1HECPM1	Not determined	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H10	Dog A1	H10A1HECPM3	H	C, TE, SXT	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H11	Dog A1	H11A1PWPM1	D	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H13	Dog A1	H13A1TBPM1	B	TE	*mrpA*, *pmfA*, *hmpA*/*hmpB*
H14	Dog A1	H14A1MCKPM1	F.2	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H14	Dog A2	H14A2MCKPM1	F.1	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H14	Dog A2	H14A2MCKPM2	F.3	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H14	Dog A2	H14A2MCKPM5	F.2	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H17	Human Hu1	H17Hu1TBPM1	G	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H17	Dog A1	H17A1HECTPM4	A.1	AMP, CN, C, TE, SXT	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H17	Dog A1	H17A1HECTPM5	A.2	AMP, CN, C, TE, SXT	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H17	Dog A1	H17A1TBPM1	C	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H18	Human Hu1	H18Hu1TBPM1	E.1	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H18	Human Hu1	H18Hu1TBPM2	E.2	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H18	Human Hu1	H18Hu1TBPM3	E.3	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H18	Human Hu1	H18Hu1TBPM4	E.4	CN, TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*
H18	Dog A1	H18A1TBPM1	E.4	TE	*mrpA*, *pmfA*, *ucaA, hmpA*/*hmpB*

Legend: *hpmA*/*hpmB,* haemolysin HpmA/HpmB; *mrpA*, mannose-resistant *Proteus*-like fimbriae (MR/P); *pmfA*, *Proteus mirabilis* fimbriae (PMF); *ucaA*, urothelial cell adhesion fimbriae (UCA/NAF); AMP, ampicillin; C, chloramphenicol; CN, gentamycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline.

Another interesting finding was that only one dog (H17A1) was colonised by several unrelated *P. mirabilis* strains (Figure 2, Table 1).

The H17 household human/dog pair (H17Hu1 and H17A1) were colonised by unrelated *P. mirabilis* stains (Figure 2, Table 1). On the other hand, the H18 household human/dog pair (H18Hu1 and H18A1) were colonised by indistinguishable and closely related *P. mirabilis* strains (89-100% Dice/UPGMA index), thus showing that *P. mirabilis* are likely shared between humans and animals living in close contact (Figure 2, Table 1). It should be noted that although the *P. mirabilis* strains from the dog H18A1 (H18A1TBPM1) and the human H18Hu1 (H18Hu1TBPM4) were considered indistinguishable by DICE/UPGMA, the resistance phenotype varied in the gentamicin-susceptibility (Figure 2, Table 1). Furthermore, at visual inspection, the second and third restriction band from the top seem to vary slightly. The detection of *P. mirabilis* colonisation in dog H18A1 required the use of tetrathionate broth while in the human H18Hu1 faecal sample this was not necessary. Both colonised humans from household H17 and H18 reported to frequently allow the colonised dogs to lick their faces.

Indistinguishable and closely related *P. mirabilis* strains (91.7-100% Dice/UPGMA index) were also shared by the two dogs living in household H14 (Figure 2, Table 1). Here, the dog H14A2 presented a higher faecal burden than the dog H14A1.

Overall, *P. mirabilis* human-dog sharing occurred in 5.5% (n= 1/18) of all included households and in 12.8% (n= 1/8) of positive households. If only the households that included dogs were to be considered, these percentages go up to 7.7% (*n*= 1/13) and 14.3% (*n*= 1/7), respectively (Figure 1). Furthermore, if the potential within-household human-dog sharing-pairs are considered, 11.1% (*n*= 1/9) or 4.2% (*n*= 1/24) of these shared *P. mirabilis* strains, depending on whether households without colonised participants are included.

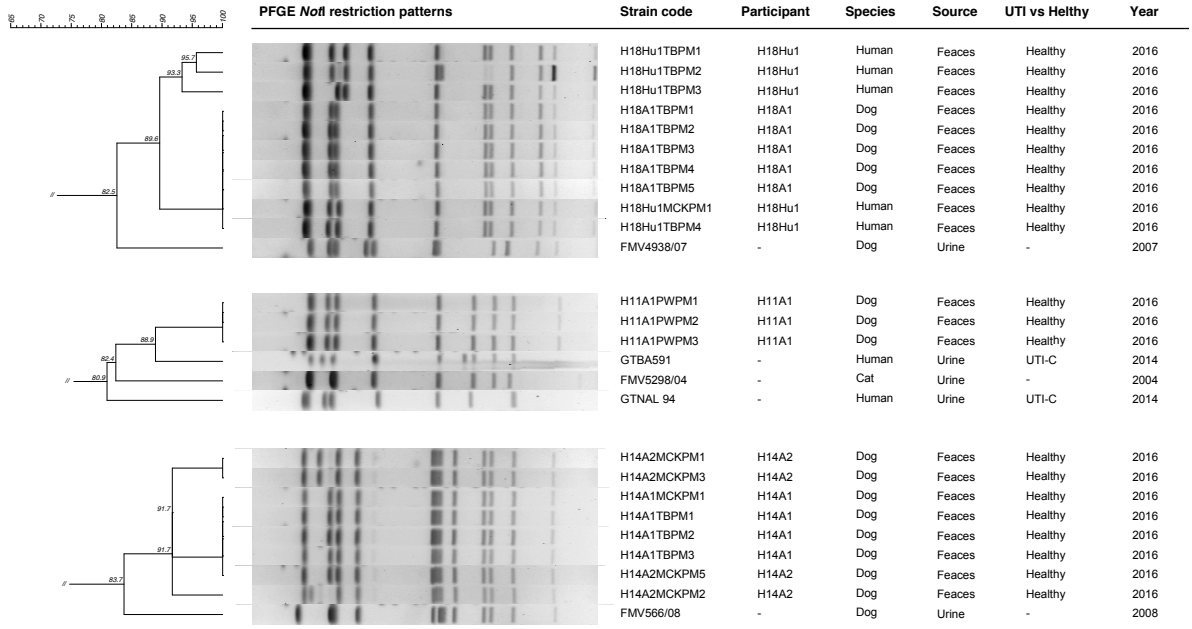
A total of 18 *P. mirabilis* strains were screened for antimicrobial resistance and for the presence of virulence genes (Table 1).

Most *P. mirabilis* (77.7%, *n*= 14/18) were fully-susceptible to all tested antimicrobials except tetracycline, for which *P. mirabilis* is intrinsically resistant. Only the dog H17A1 was colonised by a *P. mirabilis* strain with acquired resistant to three or more antimicrobial categories (Table 1).

The HmpA/HmpB hemolysin and the MR/P and PMF fimbriae codifying genes were present in all strains. Furthermore, only one strain lacked *ucaA* (Table 1). All the PFGE indistinguishable and closely related *P. mirabilis* strains also presented identical antimicrobial resistance phenotype and virulence genotype, with the exception of the H18Hu E.4 (H18Hu1TBPM4) *P. mirabilis* variant.

When comparing the faecal *P. mirabilis* isolated in this study with the previously characterised collection of clinical *P. mirabilis* from companion animals and humans with UTI (Marques et al., 2018a), faecal strains from the H11 (pulse-type D), H14 (pulse-type F) and H18 (pulse-type E) clustered with at least one uropathogenic clinical strain (Figure 3).

Figure 3 - Clusters containing faecal *P. mirabilis* strains and clinical *P. mirabilis* strains from UTI



Strain code	Participant	Species	Source	UTI vs Helthy	Year
H18Hu1TBPM1	H18Hu1	Human	Feaces	Healthy	2016
H18Hu1TBPM2	H18Hu1	Human	Feaces	Healthy	2016
H18Hu1TBPM3	H18Hu1	Human	Feaces	Healthy	2016
H18A1TBPM1	H18A1	Dog	Feaces	Healthy	2016
H18A1TBPM2	H18A1	Dog	Feaces	Healthy	2016
H18A1TBPM3	H18A1	Dog	Feaces	Healthy	2016
H18A1TBPM4	H18A1	Dog	Feaces	Healthy	2016
H18A1TBPM5	H18A1	Dog	Feaces	Healthy	2016
H18Hu1MCKPM1	H18Hu1	Human	Feaces	Healthy	2016
H18Hu1TBPM4	H18Hu1	Human	Feaces	Healthy	2016
FMV4938/07	-	Dog	Urine	-	2007
H11A1PWPM1	H11A1	Dog	Feaces	Healthy	2016
H11A1PWPM2	H11A1	Dog	Feaces	Healthy	2016
H11A1PWPM3	H11A1	Dog	Feaces	Healthy	2016
GTBA591	-	Human	Urine	UTI-C	2014
FMV5298/04	-	Cat	Urine	-	2004
GTNAL 94	-	Human	Urine	UTI-C	2014
H14A2MCKPM1	H14A2	Dog	Feaces	Healthy	2016
H14A2MCKPM3	H14A2	Dog	Feaces	Healthy	2016
H14A1MCKPM1	H14A1	Dog	Feaces	Healthy	2016
H14A1TBPM1	H14A1	Dog	Feaces	Healthy	2016
H14A1TBPM2	H14A1	Dog	Feaces	Healthy	2016
H14A1TBPM3	H14A1	Dog	Feaces	Healthy	2016
H14A2MCKPM5	H14A2	Dog	Feaces	Healthy	2016
H14A2MCKPM2	H14A2	Dog	Feaces	Healthy	2016
FMV566/08	-	Dog	Urine	-	2008

Legend: UTI-C, community patient with UTI; UTI-H, hospitalised patient with UTI.

P. mirabilis colonising the dog H11A1 clustered with two clinical strains causing UTI in human community patients in 2014 (GTBA591 and GTNAL94) and with one clinical strain isolated from a cat with UTI in 2004 (FMV5298/04). Data concerning prior history of UTI in the dog H11A1 or in his household members was not disclosed.

P. mirabilis strains from the human (H18Hu1) and the dog (H18A1) living in household H18 were 82.5% similar (Dice/UPGMA) with the *P. mirabilis* clinical strain FMV4938/07 isolated from a dog with UTI in 2007. Neither human H18Hu1 nor dog H18A1 had previous history of UTI.

Finally, the *P. mirabilis* strain shared by both dogs of the household H14 showed 83.7% similarity with a clinical strain isolated from a dog with UTI in 2008 (FMV566/08). Both dogs from H14 household were never diagnosed with UTI.

Discussion

To date only few studies have reported data on the *P. mirabilis* faecal colonisation of healthy humans and companion animals (Balish et al., 1977; Gaastra et al., 1996; Drzewiecka, 2016). Moreover, to our best knowledge this is the first study that focuses on humans and companion animals living in close contact.

P. mirabilis is an important cause of UTI and the gut is pointed as its major reservoir (Drzewiecka, 2016; Armbruster et al., 2018). In fact, several studies associate the faecal colonisation by *P. mirabilis* with cross and auto-infections (Drzewiecka, 2016).

Little is known regarding the role of healthy dogs and cats as reservoirs of *P. mirabilis*. Only 6% of feral cats from Grenada, with unknown health status, were colonised by *P. mirabilis* in

the rectum (Hariharan et al., 2010). The absence of colonised healthy domestic cats in the present study may, therefore, be explained by the small sample size. The high *P. mirabilis* colonisation of healthy dogs agrees with one early study conducted in enclosed beagles (42.8%) (Balish et al., 1977). However, another early study focussed on dogs with UTI failed to detect *P. mirabilis* in faecal samples of the control group composed by healthy dogs (Gaastra et al., 1996). The *P. mirabilis* colonisation in healthy humans ranges from 4% to 45% according to the study and to the gastro-intestinal section considered (Drzewiecka, 2016; Zilberstein et al., 2007). It is important to notice that *P. mirabilis* colonisation frequencies may vary between studies due to methodological differences. Furthermore, the normal *E. coli* faecal burden may disguise and limit the detection of less frequent bacteria. Hence, it is possible that *P. mirabilis* colonisation frequencies are being underestimated.

The growing concerns regarding the transmission of pathogenic bacteria between companion animals and humans (Damborg et al., 2016; Pomba et al., 2017) are validated by studies reporting that *E. coli* strains may be shared between companion animals and humans from the same household (Stenske et al., 2009; Naziri et al., 2016). *P. mirabilis* is a common uropathogen among cats, dogs and humans (Drzewiecka, 2016; Armbruster et al., 2018; Marques et al., 2018b); however, there is a lack of studies evaluating the colonisation of epidemiologically related companion animals and humans. One important finding from this study was the detection of within-household *P. mirabilis* sharing between human/dog and dog/dog pairs. These results suggest that dogs may be reservoirs of *P. mirabilis* to humans and vice versa but also that dogs are likely reservoirs to other dogs. These outcomes are also relevant in infection settings since dogs with UTI caused by *P. mirabilis* are known to have a significantly higher *P. mirabilis* faecal burden (Gaastra et al., 1996) which hypothetically may increase the transmission risk through direct or indirect contact. Care must be taken when interpreting these results since the faecal *P. mirabilis* strains from humans and dogs isolated in this study where not compared using the state of the art methods used nowadays, namely whole genome sequencing. Nevertheless, following the Tenover et al. (1995) criteria and considering that these strains were epidemiologically related, these findings support that humans and dogs may share *P. mirabilis* strains.

The indistinguishable *P. mirabilis* strains from the dog H18A1 and human H18Hu1 showed distinct gentamicin-susceptibility which may be related with acquisition or loss of the gentamicin-resistance mechanisms after gut colonisation. The study design used in this work does not allow to determine the transmission dynamics of *P. mirabilis*; however, since *P. mirabilis* was only detected without the use of tetrathionate broth in the colonised human (H18Hu1), it could be speculated that he had a higher *P. mirabilis* faecal burden and was more likely to transmit it. The same rational applies to the household H14 dog/dog sharing. A common source of acquisition, such as a fomite, could also have been involved in the co-colonisation of both household members. Future studies using whole genome sequencing and

preferably a longitudinal design are warranted to clarify the *P. mirabilis* transmission dynamics and colonisation persistence in humans and dogs.

E. coli within-household sharing has been found to be significantly more common among dog/human pairs that report to allow the dog to lick the human's face (Naziri et al., 2016). It was not possible to establish such association in the current study due to sample size.

The presence of several variants of the same pulse-type colonising a single host suggests that within-host *P. mirabilis* evolution may be occurring, which is a known phenomenon already reported in other bacteria (Didelot, Walker, Peto, Crook & Wilson, 2016).

Previous studies on *E. coli* found that 17%, 9.8%, 8.8% or 3.5% of households had human/dog pairs sharing indistinguishable or closely related *E. coli* clones by PFGE (94-100% PFGE profile similarity) (Johnson et al., 2008b; Stenske et al., 2009; Harada et al., 2012b; Naziri et al., 2016). The different sharing frequencies reported in these studies may be explained by the different number of participants per household included and by the different number of *E. coli* isolates typed per participant. Overall, a higher number of participant per household and a higher number of isolates per participant usually lead to the detection of a higher percentage of sharing (Johnson et al., 2008b; Stenske et al., 2009; Harada et al., 2012b; Naziri et al., 2016). It was interesting to notice that the human/dog *P. mirabilis* sharing frequency detected in this study is within a similar range as previously published for *E. coli*. On the other hand, unlike in *E. coli* studies (Stenske et al., 2009; Naziri et al., 2016), *P. mirabilis* strains were not shared across-households. Likelly, this was related to the small sample size.

Johnson et al. (2008b) reported that adult humans shared *E. coli* strains more frequently with cats than with dogs. Regarding *P. mirabilis* the opposite was noted.

The detection of multidrug-resistant CMY-2-producing *P. mirabilis* is increasing in companion animals with UTI living in the same geographic region where the present study was conducted (Marques et al., 2018b). CMY-2-like β-lactamases are also important in human infection in Europe (D'Andrea et al., 2011). Furthermore, antimicrobial use in the prior year is a known risk factor for the gut colonisation by third-generation cephalosporin-resistant bacteria (Belas et al., 2014; Karanika, Karantanos, Arvanitis, Grigoras & Mylonakis, 2016). Despite the inclusion of participants that underwent antimicrobial use in the prior year, third-generation cephalosporin and/or meropenem-resistant *P. mirabilis* were not detected. Although the sample size from the current study is a limitation that could explain this result, this is considered a positive finding.

The gut of catheterised humans with UTI is usually colonised by the index uropathogenic *P. mirabilis* strain (Mathur et al., 2005; Drzewiecka, 2016). The clonal relatedness between *P. mirabilis* clinical strains from individuals with UTI and *P. mirabilis* strains colonising healthy individuals is poorly studied. In the present study, some *P. mirabilis* strains from faecal samples (from household H11, H14 and H18 colonised participants) clustered with UTI clinical strains from unrelated patients with UTI. Regarding the household H14 and H18, none of the colonised participants have had prior UTI in their life. These findings suggest the role of the gut as

reservoir of uropathogenic *P. mirabilis* in healthy individuals. The detection of *P. mirabilis* colonising a dog that clustered with clinical *P. mirabilis* isolated from human community-associated UTI also points to the possible role of dogs as reservoirs to humans. Regretfully, it was not possible to obtain epidemiological data on this dog concerning prior UTI or contact with infected individuals. Furthemore, future studies using whole genome sequencing are warrented to confirm the clonal reladness between these strains.

Notably almost all *P. mirabilis* colonising healthy companion animals and humans codified for HmpA/HmpB hemolysin and UCA/NAF, PMP and MR/P fimbriae, which are important virulence factors for UTI (Armbruster et al., 2018).

In conclusion, to our knowledge, this study showed for the first time that co-living dogs and humans may share closely related *P. mirabilis* strains in the gut that may also be closely related with clinical strains from UTI. Taken together with the high frequency of *P. mirabilis* faecal colonisation in dogs, these findings underline the likely role of dogs in the transmission cycle of uropathogenic *P. mirabilis*. Although this study has a limited sample size and used PFGE instead of whole genome sequencing, it provides new epidemiological data that has important implications in *P. mirabilis* dissemination control. Until further studies are conducted, risk factors that have been associated with higher *E. coli* human/dog sharing (e.g. dog liking and kissing the face) (Naziri et al., 2016) should be assumed to also contribute for *P. mirabilis* sharing between pets and humans.

Chapter 6

Discussion, conclusions and future perspectives

6.1 General discussion

The discovery of antimicrobials revolutionised the treatment of infections in human and veterinary medicine (Prescott, 2017). However, the use of antimicrobials led to the acquisition of antimicrobial resistance by bacteria with consequent treatment failure (Prescott, 2017). Due to the limited number of antimicrobials available nowadays and the lack of new antimicrobial class development, the rising of antimicrobial resistance is a major concern in veterinary and human medicine (Prescott, 2017). Research on antimicrobial resistance epidemiology is essential to support antimicrobial stewardship programmes and to limit further antimicrobial resistance dissemination.

Antimicrobial resistance of uropathogenic bacteria in companion animals

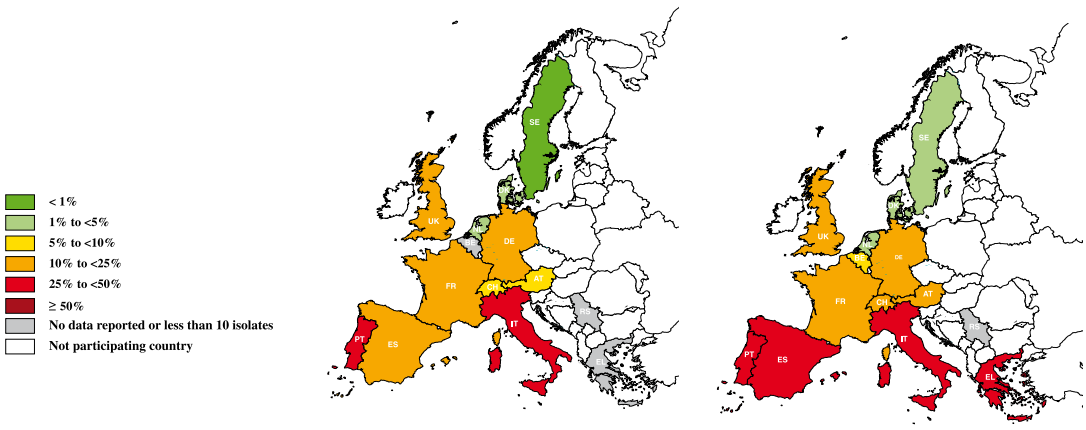
UTIs are among the most common infections diagnosed in dogs and humans and therefore are a frequent reason for antimicrobial prescription (Harding & Ronald, 1994; Schappert & Rechtsteiner, 2011; Weese et al., 2011; ECDC, 2013). This infection is also an important cause of antimicrobial use in groups of cats with higher frequency of UTI, such as elderly cats (Lekcharoensuk et al., 2001). Bacterial antimicrobial resistance frequencies may vary over time and the with geographical location (Dorsch et al., 2015; ECDC, 2017). Therefore, global surveillance of the antimicrobial resistance trends in small animal veterinary medicine is essential to guide the rational use of antimicrobials.

The study included in the Chapter 3.1 from this thesis aimed at obtaining an overview of the European geographic distribution of antimicrobial resistance in bacteria causing UTI in companion animals following the example of the EARS-Net on reporting antimicrobial resistance of invasive bacteria from humans over Europe (ECDC, 2017). Although there are several published studies reporting data on antimicrobial resistance in bacteria causing UTIs in companion animals, results are not easily comparable due to the use of different inclusion criteria (e.g. studied population, time frame, type of UTI) (Normand, et al., 2000a; Normand et al., 2000b; Meunie et al., 2004; Authier et al., 2006; Bailiff et al., 2006; Pedersen et al., 2007; Ball et al., 2008; Harada et al., 2012a; Hall et al., 2013; Kroemer et al., 2014; Beever et al., 2015; Dorsch et al., 2015; Wong et al., 2015; Moyaert et al., 2017; Rampacci et al., 2018;

Teichmann-Knorrn et al., 2018). Furthermore, the few studies that included samples from different countries usually present data as total antimicrobial resistance frequencies (Meunier et al., 2004; Kroemer et al., 2014; Moyaert et al., 2017).

Reporting the geographic distribution of antimicrobial resistance is useful to identify the locations where efforts should be made to improve awareness and implement new strategies aiming to decrease antimicrobial resistance. The multicentric study here presented, included samples from 16 veterinary microbiology laboratories from 14 European countries and showed striking geographical differences on *E. coli* and *P. mirabilis* antimicrobial resistance between Northern (Denmark and Sweden) and Southern (Italy, Greece, Portugal and Spain) European countries. It is interesting to notice that the European geographic distribution of antimicrobial resistance in uropathogenic *E. coli* from companion animals closely resembles that from EARS-Net reports (Chapter 1-Figure 5 and Chapter 6-Figure 1).

Figure 1 – *E. coli* resistance to third-generation cephalosporins and fluoroquinolones in companion animals with UTI by country



Legend: Results from the study presented at Chapter 3.1; Left image, third-generation cephalosporins; Right image, fluoroquinolones.

The use of different antimicrobial testing methods and interpretation criteria in some countries is a study limitation that could have biased these findings. However, the wide differences detected between some countries (e.g. 2.88% and 48.15% amoxicillin/clavulanate-resistance in Denmark and Portugal, respectively) are likely a result from true geographic differences. This limitation is also faced by the EARS-Net reports (ECDC, 2017) and should not, in our view, restrain researchers to gather such important epidemiological data.

The lack of harmonisation regarding the testing and reporting of antimicrobial susceptibility of *Staphylococcus* spp. was profound and strongly limited the analysis of methicillin-resistance. Identification of staphylococci to species and testing of the adequate methicillin surrogate is essential; however, data was not always provided. Efforts should be made in the future to harmonise veterinary microbiology methods across Europe to ensure proper testing and

reporting. Hopefully, the VetCAST (Toutain et al., 2017) will play a pivotal role in this issue by being a opensource resource to veterinary microbiologists.

The selection of antimicrobial resistance is a complex and multifactorial process (Prescott, 2017). Different antimicrobial use policies in each European country may contribute to the lower *E. coli* and *P. mirabilis* antimicrobial resistance in the Northern (Denmark and Sweden) countries when compared to Southern countries (Italy, Greece, Portugal and Spain). Nevertheless, antimicrobial use data about companion animals in different European countries is still scarce (Pomba et al., 2017) which limits further conclusions. The use of culture and antimicrobial susceptibility testing prior to prescribing avoids the misuse of antimicrobials and therefore may also lead to a decrease in local antimicrobial selective pressure. A study conducted in Europe found, for instance, that veterinarians in Sweden were 15.64 times more likely to conduct antimicrobial susceptibility testing to guide antimicrobial choice than in Spain (de Briyne, Atkinson, Pokludová, Borriello & Price, 2013).

Since for most countries from this study samples were obtained from a single veterinary microbiology laboratory, these findings may not be representative of the entire country. Despite this limitation, these results should prompt the Southern countries to evaluate the geographic distribution and temporal trends in antimicrobial resistance within each country.

The little updated information available regarding antimicrobial resistance in companion animals with UTI from Portugal (Féria et al., 2000; Féria et al., 2002), led to the second study here presented in Chapter 3.2. The first aim of this study was to determine the antimicrobial resistance temporal trends in uropathogenic bacteria over 16 years. Notably, a significant increase in Enterobacteriaceae antimicrobial resistance to the main antimicrobials used for UTI treatment in veterinary medicine was observed in Portugal (Lisbon). The high frequency of antimicrobial resistance detected in Enterobacteriaceae in recent years was worrisome. Since the antimicrobial classes used in veterinary medicine are common to those used in human medicine (Chapter 1-Table 3), these results are also important for human health.

The fact that the two studies presented in Chapter 3 relied on samples sent to the laboratory based on the clinician judgment could be considered a bias towards resistance (de Briyne et al., 2013). Another European study (Portugal not included) showed extremely high antimicrobial susceptibility frequencies among bacteria from companion animals with UTI obtained between 2008 and 2010 (Moyaert et al., 2017). Although apparently contradicting our results, the fact that Moyaert et al. (2017) study excluded samples from companion animals with chronic diseases or with previous history of antimicrobial use in the past 4 weeks may have biased these results towards susceptibility. Nevertheless, both studies have important epidemiological value. The Moyaert et al. (2017) study is more useful to guide empirical antimicrobial treatment since it likely included a higher number of simple uncomplicated UTIs. On the other hand, the Chapter 3.1 study shows the real therapeutic limitations that veterinarians face nowadays in overall UTIs according to each country. Furthermore, the fact

that Moyaert et al. (2017) study reports to earlier years may also have contributed to this difference.

In the study presented in the Chapter 3.2, the clonal lineages of methicillin-resistant staphylococci, ampicillin-resistant and HLGR enterococci and third-generation cephalosporin-resistant *E. coli* were determined by MLST. The significant increase in the detection of MDR Enterobacteriaceae, including *E. coli*, noted in Portugal (Lisbon) creates important therapeutic limitations. The two main MDR *E. coli* clonal lineages detected in this study (ST648 and O25b:H4-B2-ST131) are worldwide disseminated in human infection and mostly associated with CTX-M-producing strains (Coque et al., 2008; Doi et al., 2017; Poirel et al., 2018). Interestingly, previous studies about the population structure of *E. coli* have shown that some ST131 and ST648 *E. coli* strains isolated from companion animals cluster with *E. coli* strains from humans (Pomba et al., 2014b; Fernandes et al., 2018b). Therefore, the significant increase in MDR CMY-2-producing *E. coli* ST648 detected in Portugal (Lisbon) is worrisome. Furthermore, although ampicillin-resistant and/or HLGR Enterococci, MRSA and MRSE were not as frequent, they also belonged to important clonal lineages previously associated to human infection (Leavis et al., 2006; Kuch et al., 2012; Rolo et al., 2012; Aires-de-Sousa, 2017; Lee, Pang, Abraham & Coombs, 2018; Torres et al., 2018). Overall, this study showed that bacterial species causing UTI in companion animals are becoming more resistant and some MDR strains belong to clonal lineages associated to human infection. Studies using WGS are needed to clarify the true zoonotic nature of these strains; nevertheless, the role of companion animals as reservoirs should not be neglected and is considered a public health concern.

Molecular epidemiology of K. pneumoniae and P. mirabilis causing UTI

The results from the two studies presented in Chapter 3 were in line with previous reports showing that *E. coli* predominates among dogs and cats with UTI (Bush, 1976; Wooley & Blue, 1976; Forrester et al., 1999; Gupta et al., 1999; Hess et al., 2000; Ling et al., 2001; Cohn et al., 2003; Kahlmeter & ECO.SENS, 2003; Pressler et al., 2003; Torres et al., 2005; Bailiff et al., 2006; Litster et al., 2007b; Mayer-Roenne et al., 2007; Passmore et al., 2008; Martinez-Ruzafa et al., 2012; Dorsch et al., 2015; Flores-Mireles et al., 2015; Moyaert et al., 2017). Although less frequent, *P. mirabilis* is the second most common Enterobacteriaceae isolated in companion animals with UTI, particularly in dogs (Wooley & Blue, 1976; Lekcharoensuk et al., 2001; Ling et al., 2001; Cohn et al., 2003; Bailiff et al., 2006; Bailiff et al., 2008; Litster et al., 2007b; Moyaert et al., 2017). In humans, *P. mirabilis* is an important nosocomial pathogen causing CAUTI (Szabo & Paterson, 2002; Manos & Belas, 2006; Drzewiecka, 2016); nevertheless, it has deserved little attention in companion animals. In Portugal (Lisbon), the study from Chapter 3.2 showed a significant increase in *P. mirabilis* resistance to several CIAs over time that was associated with an increase in the detection of MDR CMY-2-producing

P. mirabilis. The frequent detection of CMY-2 among third-generation cephalosporin-resistant *P. mirabilis* is in line with previous reports about companion animals (Dierikx et al., 2012; Hordijk et al., 2013; Harada et al., 2014; Schultz et al., 2017b). The population structure of these strains was analysed by PFGE in the study from Chapter 4.2 and showed that several MDR CMY-2-producing *P. mirabilis* from companion animals clustered together. These MDR CMY-2-producing strains were resistant to 5-9 antimicrobial categories. Therefore, their possible dissemination is worrisome and should be monitored in the future. Interestingly, the detection of CMY-2-producing *P. mirabilis* is also being increasingly reported among human infections (Aragón et al., 2008; D'Andrea et al., 2011; Song et al., 2011; Miró et al., 2013a; Aogáin et al., 2016).

K. pneumoniae is currently in the spot light due to their role in the dissemination of ESBLs and carbapenemases (Brisse et al., 2006; ECDC, 2017; Navon-Venezia et al., 2017; Martin & Bachman, 2018). In women, *K. pneumoniae* is the second most frequent Enterobacteriaceae causing UTI (Gupta et al., 1999) while in dogs and cats it seems less frequent. Due to the limited number of *K. pneumoniae* from companion animals with UTI included in the study from Chapter 3.2 and Chapter 4.1, it was not possible to evaluate its antimicrobial resistance trends over time. Nevertheless, it was surprising that more than 50% of isolates were MDR ESBL/AmpC-producers. The main ESBL gene detected in *K. pneumoniae* strains from companion animals with UTI was the $bla_{\text{CTX-M-15}}$, which is in line with previous reports in humans and animals (Ewers et al., 2014b; Calbo & Garau, 2015; Harada et al., 2016; Navon-Venezia et al., 2017).

The high detection of MDR Enterobacteriaceae, including *K. pneumoniae* and *P. mirabilis*, causing UTIs in companion animals is worrisome because it frequently leads to the need to prescribe off-label antimicrobials under the cascade principals (EMA, 2018). Besides important ethical issues, this use creates additional resistance selective pressure to an already limited number of antimicrobials available nowadays (EMA, 2018). The urgent implementation of antimicrobial stewardship programmes in veterinary medicine is essential to improve the judicious use of antimicrobials (Lloyd & Page, 2018). Following the 5 Rs (responsibility, reduction, replacement, refinement and review) of the antimicrobial stewardship framework (Lloyd & Page, 2018), there is a need to test new therapeutic approaches to address UTIs caused by MDR bacteria in companion animals. Recently, following the example of studies from humans (Lagacé-Wiens et al., 2006; Beytur et al., 2015), a pilot study was conducted to evaluate the use of amoxicillin/clavulanate for the treatment of a cat with UTI by a MDR CTX-M-15-producing *K. pneumoniae* (Marques, Belas, Vetpoint Team, Salas & Pomba, 2017). Although clinical cure was not achieved and further studies are needed, a decrease in bacteriuria during treatment was observed.

The close contact between companion animals and humans in modern society is viewed as an important route for the dissemination of antimicrobial resistant bacteria (Guardabassi et al.,

2004; Damborg et al., 2016; Pomba et al., 2017). Furthermore, companion animals with UTI have high bacterial loads in urine and frequently present polyakuria (Gaastra et al., 1996; Bartges et al., 2004; Weese et al., 2011), which likely enhances the risk of dissemination. There is very little information regarding the molecular epidemiology of *P. mirabilis* from companion animals with UTI (Schultz et al., 2015; Schultz et al., 2017b) and only recently studies started to focus on *K. pneumoniae* (Chapter 1-Table 6). In the studies presented in the Chapter 4, *K. pneumoniae* and *P. mirabilis* from companion animals and humans with UTI were characterised for their population structure, antimicrobial resistance and virulence genotype. Previous studies about *K. pneumoniae* and *P. mirabilis* strains of companion animal origin only rarely included isolates from humans (Stolle et al., 2013; Ewers et al., 2014b; Schultz et al., 2015; Wohlwend et al., 2015; Schultz et al., 2017b).

Interestingly, the main antimicrobial resistant genes detected in *K. pneumoniae* and *P. mirabilis* from companion animals and humans with UTI were similar. Regarding *E. coli*, some studies suggest that different CTX-M ESBLs predominate in strains from animals or humans (Coque, Baquero & Canton, 2008; Ewers et al., 2012). In the third-generation cephalosporin-resistant *K. pneumoniae* studied in Chapter 4.1, CTX-M-15 predominated regardless of its origin. Furthermore, other antimicrobial resistant genes, such as bla_{TEM}, bla_{OXA-1}, bla_{CMY-2}, bla_{DHA-1}, *qnrB*, *aac(6')-Ib*, *sul1*, *sul2* and *tet(A)*, were also detected in *K. pneumoniae* strains of both origins. A similar overlap was observed in *P. mirabilis* from humans and companion animals regarding bla_{TEM}, *qnrD*, *aphAI-IAB*, *sul1*, *sul2* and *dfrIa.*

A positive finding from Chapter 4 studies was the lack of carbapenem-resistance due to the production of carbapenemases in *K. pneumoniae* and *P. mirabilis* from humans and companion animals. So far the isolation of carbapenemase-producing *K. pneumoniae* in companion animals still remains a sporadic finding (Stolle et al., 2013; Schmiedel et al., 2014; González-Torralba set al., 2016; Pulss et al., 2018). On the other hand, carbapenemase-producing *K. pneumoniae* have been extensively detected in human patients from Portugal (ECDC, 2017) (Chapter 1-Table 7).

In the study presented in Chapter 4.1, the population structure of the clinical *K. pneumoniae* strains from companion animals and humans with UTI was characterised by PFGE and MLST. PFGE has the advantage of being a more discriminatory typing method while MLST allows the comparison of results with published data worldwide (Hansen et al., 2002; Vimont et al., 2008). Since 2012, the number of studies typing *K. pneumoniae* from companion animals by MLST has increased; however, data is mainly focussed on ESBL/carbapenamase-producers (Chapter 1-Table 6). In this study, *K. pneumoniae* from companion animals with UTI belonged predominantly to ST15 regardless of being or not an ESBL or AmpC-producer. The predominance of ST15 among CTX-M-15-producing *K. pneumoniae* is in line with reports from other countries (Chapter 1-Table 6) and our results agree with the hypothesis that companion animals may be reservoirs of this high-risk clonal lineage (Ewers et al., 2014b). Other high-risk

clonal lineages previously associated with the dissemination of ESBLs and carbapenemases in humans (Navon-Venezia et al., 2017) were also detected in companion animals with UTI in Portugal (Lisbon), namely ST11, ST37 and ST147. In fact, it should be noted that the third-generation cephalosporin-resistant *K. pneumoniae* from companion animals in this study belonged mainly to high-risk clonal lineages to humans. These *K. pneumoniae* high-risk clonal lineages have also been found in companion animals from other European and Asian countries, with ST147 being the second most disseminated after ST15 (Chapter 1-Table 6). Notably, most of the MDR *K. pneumoniae* clonal lineages here detected in companion animals with UTI (ST15, ST11, ST147 and ST348) are also among the most reported in humans from Portugal, including carbapenemase-producers (Chapter 1-Table 7).

The *K. pneumoniae* strains from humans with UTI included in Chapter 4.1 showed higher diversity by PFGE and MLST, which may point to different clonal epidemiology between humans and companion animals. Similar findings were also reported by Poirel et al. (2013) and Wohlwend et al. (2015). Since UTIs caused by *K. pneumoniae* are more common in humans than in companion animals (Gupta et al., 1999; Moyaert et al., 2017) this higher diversity may suggest that *K. pneumoniae* are less adapted to the urinary tract of dogs and cats. In fact, as pointed by Ewers et al. (2014b), it is still unclear whether *K. pneumoniae* ST15 clonal lineage is truly zoonotic or if a separate host microevolution is in place.

The PFGE analysis of *P. mirabilis* from UTIs conducted in Chapter 4.2 showed a high clonal diversity and that a big number of PFGE clusters included strains (43.6%) isolated from companion animals and humans. Furthermore, some *P. mirabilis* strains from cats had 92%-100% similarity to strains isolated from humans, thus suggesting that some *P. mirabilis* strains may be able to cause UTI in both hosts.

The use of WGS offers maximum discrimination between bacterial strains by comparing almost the entire bacterial genome (Struelens & Brisse, 2013; Quainno et al., 2017). The fact that the phylogenetic relatedness of *K. pneumoniae* and *P. mirabilis* strains was only characterised by PFGE is considered a study limitation because it restricted the recognition of a definite link between companion animals and humans. Nevertheless, the detection of *K. pneumoniae* and *P. mirabilis* PFGE clusters containing strains of both origins is noteworthy and should encourage further investigations. The lack of an available *P. mirabilis* MLST typing scheme also strongly limits the comparison of results regarding clonality worldwide and is likely one of the reasons why the global *P. mirabilis* clonal epidemiology is fairly unknown.

Several *K. pneumoniae* and *P. mirabilis* virulence genes were very abundant regardless of the strain origin. The high virulence gene frequencies detected in *P. mirabilis* could be expected based on several reports from humans and early studies from companion animals (Old & Adegbola, 1982; Mobley & Chippendale, 1990; Swihart & Welch, 1990b; Bijlsma et al., 1995; Gaastra et al., 1996; Sosa et al., 2006; Cestari et al., 2013; Kuan et al., 2014). Some of the virulence genes tested in *K. pneumoniae*, namely *fimH-1*, *mrkD* and *entB,* are thought to be

ubiquitous and therefore the results obtain in Chapter 4.1 were not a surprise (Podschun & Ullmann, 1998; Paczosa & Mecsas, 2016; Martin & Bachman, 2018). All *K. pneumoniae* included in this study had virulence genotypes characteristic of "classic" *K. pneumoniae* (Shon et al., 2013; Paczosa & Mecsas, 2016). Nevertheless, some virulence genes associated with invasive *K. pneumoniae* (Shon et al., 2013; Holt et al., 2015; Paczosa & Mecsas, 2016), such as the yersiniabactin, were also detected in strains from companion animals belonging to high-risk clonal lineages. The absence of hypervirulent *K. pneumoniae* was expected since only recently it was reported in a human infection from Portugal (Pereira et al., 2017).

The similarities noted between the *K. pneumoniae* or *P. mirabilis* strains isolated from companion animals and humans with UTI raise concerns regarding its possible animal-to-human transmission or vice versa. Nonetheless, it should be noted that a significant number of *K. pneumoniae* and *P. mirabilis* strains from companion animals with UTI were unrelated with strains isolated from humans. Additionally, differences were also noted in some antimicrobial resistance gene and virulence gene frequencies. One possible explanation is the fact that although companion animals and humans live in close contact, they are also exposed to different bacterial sources (e.g. food, living environment and lifestyle, etc). Furthermore, the inclusion of *K. pneumoniae* and *P. mirabilis* strains from companion animals and humans collected in different time frames may have limited the detection of additional epidemiological links. It could also be speculated that some *K. pneumoniae* and *P. mirabilis* strains may be more adapted to one of the host species through separate evolution. Future studies using WGS are warranted to clarify these questions. Since the adhesion to the urinary tract is an essential step for UTI (Flores-Mireles et al., 2015), the comparison of adhesin sequences that are known to contribute for UTI could disclose the existence of host-specific adaptations or, on the contrary, show similar and zoonotic features.

Sharing of K. pneumoniae and P. mirabilis between humans and companion animals

Most studies characterising the population structure of *K. pneumoniae* and *P. mirabilis* isolated from companion animals are focussed on clinical strains from a wide range of infections (Schultz et al., 2015; Schultz et al., 2017b) (Chapter 1-Table 6). The role of healthy companion animals as reservoirs of *K. pneumoniae* and *P. mirabilis* has seldom been addressed (Balish et al.,1977; Gaastra et al., 1996; Hariharan et al., 2010; Shariff et al., 2017) and there is a lack of information regarding the population structure of colonising strains. However, the number of healthy companion animals surely surpasses those with infections and therefore their role as reservoirs of *K. pneumoniae* and *P. mirabilis* should be investigated. In Chapter 5, the *K. pneumoniae* and *P. mirabilis* gut colonisation of healthy humans and companion animals living together was evaluated regarding sharing and population structure.

Only dogs and humans were found to be colonised by both bacteria. The *K. pneumoniae* and *P. mirabilis* gut colonisation frequencies detected in dogs were in line with the Balish et al.

(1977) study. The gut is thought to be the reservoir of uropathogenic bacteria (Podschun & Ullmann, 1998; Drzewiecka, 2016; Johnson et al., 2016; Martin et al., 2016) and therefore the lower frequency of *K. pneumoniae* and *P. mirabilis* in the gut when compared to *E. coli* may explain the predominance of this latter as a cause of UTI.

Considering the high frequency of CMY-2-producing *P. mirabilis* and ESBL-producing *K. pneumoniae* detected in recent years in companion animals with UTI from Portugal (Lisbon), it was surprising not to detect gut colonisation by third-generation cephalosporin-resistant *P. mirabilis* or *K. pneumoniae* in dogs and cats. Likewise for humans, considering the EARS-Net reports about human infection by third-generation cephalosporin and carbapenem-resistant *K. pneumoniae* (ECDC, 2017). The fact that these studies relied on samples from healthy dogs and humans without antimicrobial treatment in the prior month may have biased the results towards the detection of susceptible strains.

The *K. pneumoniae* strains detected in the gut of companion animals and humans belonged to a diverse number of clonal lineages. This finding is consistent with the high *K. pneumoniae* diversity detected in the study presented in Chapter 4.1 and was also initially noted by Doumith et al. (2005).

Considering the frequency and dissemination of the *K. pneumoniae* high-risk clonal lineage ST15 in companion animal and human infections (Chapter 1-Table 6 and Chapter 1-Table 7) (Navon-Venezia et al., 2017), it was surprising not to detected this clonal lineage colonising healthy individuals. The same could be said about other frequently detected *K. pneumoniae* high-risk clonal lineages detected in humans from Portugal (Chapter 1-Table 7) and worldwide (Navon-Venezia et al., 2017) such as ST11 and ST147. Studies on *K. pneumoniae* are usually focussed on resistant strains, such as ESBL or carbapenemase-producers, and therefore data on susceptible strains is scarce (Martin et al., 2016; Martin & Bachman, 2018). The fact that only susceptible *K. pneumoniae* strains to third-generation cephalosporins were typed in Chapter 5.1 may explain these unexpected results. Importantly, the high-risk clonal lineage ST17 (Navon-Venezia et al., 2017), which is frequently associated with the production of ESBLs, was detected in the gut of dogs and humans. When searching the Institut Pasteur Bigsdb (2018) for the *K. pneumoniae* clonal lineages detected in the gut of dogs, it becomes clear that these clonal lineages have been mainly associated with human infection (Chapter 6-Table 1). This is an important finding from the Chapter 5.1 study because it suggests that healthy dogs may be reservoirs of *K. pneumoniae* to humans.

Due to the lack of an MLST scheme, such epidemiological conclusions could not be drawn regarding *P. mirabilis* clonal lineages. *P. mirabilis* colonising the gut of dogs and humans also showed high diversity by PFGE as seen in Chapter 4.2 regarding uropathogenic strains.

Previous studies on the gut colonisation and sharing of Enterobacteriaceae between humans and companion animals have been mainly focussed on *E. coli.* These studies have found that dogs may share identical *E. coli* strains with humans living within the same household and

across non-related households (Johnson & Clabots, 2006; Johnson et al., 2008a; Johnson et al., 2008b; Stenske et al., 2009; Harada et al., 2012b; Naziri et al., 2016; Damborg et al., unpublished data[13]). To our best knowledge, the studies presented in Chapter 5 are the first reports regarding the faecal sharing of *K. pneumoniae* and *P. mirabilis* between healthy humans and companion animals living in close contact. These two studies seem to support the hypothesis that *K. pneumoniae* and *P. mirabilis* strains may be shared between healthy humans and dogs living in close contact as previously reported for *E. coli*.

Table 1 – *K. pneumoniae* clonal lineages detected in the gut of dogs in Chapter 5.1

Sequence type	Host (Source or Infection)
ST17	Human (bacteraemia /blood, sputum, sepsis, urine and UTI, pneumonia, diarrhoea and faecal carriage, nose and throat swabs); Pig (pneumonia[a]); Raw cow milk[a].
ST188	Human (bacteraemia, UTI[a]); Dog (swab teachea[a]); Rat (lung tissue); Retail meat[a].
ST281	Human (urine, antral biopsy[a]).
ST252	Human (blood, urine and UTI[a], carriage[a]).
ST423	Human (trachea aspirate, UTI[a,b], skin[a], cerebrospinal fluid[a]).
ST1093	Human (pneumonia/blood[a]).
ST1241	Human (blood).
ST3398	Newly described in this study.
ST3399	Newly described in this study.

Legend: Information from: Institut Pasteur Bigsdb (2018) and [a]Bowring, Fahy, Morris and Collins (2017); Davis et al. (2015); Diab et al. (2017); Ewers et al. (2014b); Gomez-Simmonds et al. (2015); Ito et al. (2015); Papagiannitsis et al. (2015); Rodrigues et al. (2017); da Silva et al. (2018); Garza-Ramos et al. (2018). [b] Also detected at the study presented in Chapter 4.1.

Interestingly, within and across-household *K. pneumoniae* sharing was noted between humans and dogs. The *K. pneumoniae* strains shared by the three participants from different households belonged to the high-risk clonal lineage ST17. These ST17 *K. pneumoniae* strains shared indistinguishable PFGE pulse-types but had slightly different antimicrobial resistance phenotypes. Since the three households were epidemiologically related it could be hypothesised that the acquisition of different antimicrobial resistance genes occurred after the direct or indirect dissemination of this strain to all hosts. However, it is also plausible that this high-risk ST17 strain is more disseminated and was independently acquired by each participant. It is intriguing that, to our knowledge, the *K. pneumoniae* ST17 has only been recently reported once in humans from Portugal (Chapter 1-Table 7). One possible explanation

[13] Available as a preprint at bioRxiv at https://www.biorxiv.org/content/early/2018/04/17/302885.

is that this clonal lineage is more disseminated in community than in hospital and health-care settings.

The household H15 is an interesting case of within-household *K. pneumoniae* sharing that highlights the need of future studies to clarify the 1) *K. pneumoniae* colonisation dynamics between household members; 2) the risk factors associated with sharing; and 3) the potential sources/routes of *K. pneumoniae* transfer. Interestingly, *E. coli* colonisation shows a complex transmission dynamics where certain strains circulate and are intermittently detected among humans and companion animals from the same household (Johnson & Clabots, 2006).

The household H18 included one human and one dog that shared the same *P. mirabilis* strain, according to PFGE, but with distinct susceptibility to gentamicin. Although the Bionumeric software rendered these strain as undistinguishable, at visual examination a slight difference between the second and third upper restriction band seems to exist. These small size differences may be related with acquisition or loss of gentamicin-resistance mechanisms or other mobile genetic elements during colonisation. Ideally, WGS should be conducted in the future to compare the entire *P. mirabilis* genome of these strains and clarify their clonal relatedness.

Companion animals and humans make part of the dissemination cycle of pathogenic and antimicrobial resistant bacteria through direct and indirect contact (Prescott, 2017). If a higher gut colonisation would lead to higher chances of transmission, it could be speculated that dog-to-human and human-to-dog transmission likely occurred regarding in the *K. pneumoniae* and *P. mirabilis* studies, respectively. However, since the *K. pneumoniae* detected in the gut of dogs have been mainly previously associated with human infection (Chapter 6-Table 1), the acquisition of *K. pneumoniae* from human sources could be suggested. It should also be noted that, the clonal epidemiology of *K. pneumoniae* from healthy dogs is so far understudied, and therefore the most frequent strains colonising companion animals still need to be determined worldwide. Finally, a common source of *K. pneumoniae* and *P. mirabilis* dissemination could also have occurred.

When comparing the colonising *K. pneumoniae* and *P. mirabilis* strains, by PFGE, with the clinical strains from patients with UTI studied in Chapter 4, some strains clustered together using a clustering cut-off of 80%. Regarding *P. mirabilis*, the strain colonising dog H11A1 is noteworthy because it shows only a two band difference and 88.9% similarity from a strain isolated from a human patient with UTI (GTBA591). The same is true for the *K. pneumoniae* strain isolated from the dog H10A1 which was 92.3% similar with a strain isolated from a hospital patient with UTI (PC25/15B) belonging to ST423. Although WGS is needed to evaluate the relatedness between these strains, these findings seem to suggest that healthy dogs may be reservoirs of uropathogenic *K. pneumoniae* and *P. mirabilis*.

6.2 Conclusions

The studies conducted under this thesis contributed with important epidemiological findings to small animal veterinary medicine and public health, namely:

Antimicrobial resistance of uropathogenic bacteria in companion animals

There are significant differences in the antimicrobial resistance frequencies, including against CIAs, of uropathogenic bacteria from companion animals across Europe. The major differences, were detected between Northern (Denmark and Sweden) and Southern (Italy, Greece, Portugal and Spain) European countries. In Portugal (Lisbon), there was an increasing trend in uropathogenic Enterobacteriaceae resistance to the main antimicrobials used for UTI treatment in small animal veterinary medicine. The significant increase in detection of MDR bacteria is worrisome since it leads to bigger therapeutic limitations. The detection of high-risk clonal lineages harbouring clinically relevant antimicrobial resistant mechanisms, such as third-generation cephalosporin-resistant *E. coli* O25b:H4-B2-ST131, CC23 and ST648, MRSA CC5, MRSE CC5, HLGR *E. faecalis* CC6, and ampicillin/HLGR *E. faecium* CC17*,* highlights the role of dogs and cats with UTI in its dissemination.

Molecular epidemiology of *K. pneumoniae* and *P. mirabilis* causing UTI

The ST15 is the most common *K. pneumoniae* clonal lineage causing UTI in companion animals from Portugal (Lisbon) and is frequently MDR and CTX-M-15-producer. Furthermore, third-generation cephalosporin-resistant *K. pneumoniae* from companion animals with UTI mainly belong to high-risk clonal lineages to humans. Regarding *P. mirabilis*, a high number of *P. mirabilis* from companion animals with UTI cluster in PFGE analysis with strains isolated from human patients, some with >90% similarity. *K. pneumoniae* and *P. mirabilis* from companion animals and humans harboured similar antimicrobial resistance determinants and virulence genes. Such similarities highlight the possible role of companion animals in the dissemination and as reservoirs of uropathogenic *K. pneumoniae* and *P. mirabilis*.

Sharing of K. pneumoniae and P. mirabilis between humans and dogs

To our best knowledge, the possibility for *K. pneumoniae* and *P. mirabilis* sharing between healthy humans and dogs living in close contact was here demonstrated for the first time. Dogs also seem to be reservoirs of *K. pneumoniae* clonal lineages associated with human infection which supports their role as reservoirs. The detection of some faecal *K. pneumoniae* and *P. mirabilis* strains that were closely related to uropathogenic clinical strains is also an important finding from these studies.

Taken together, the results from this thesis are in line with the need of a One Health approach.

6.3 Future perspectives

The works here presented point to some research fields that need be further explored in the in the future.

Multicentric surveillance studies and antimicrobial stewardship.

Considering the wide geographical differences detected in antimicrobial resistance in Europe, studies on other types of infection in companion animals are needed. Ideally, future studies should pursue the characterisation of the bacterial clonal lineages affecting companion animals and their antimicrobial resistance mechanisms in order to also evaluate the dissemination of pathogenic bacterial clonal lineages and antimicrobial resistance genes.

Furthermore, the development of antimicrobial stewardship programmes suitable to small animals veterinary medicine are urgent, especially in the Southern European countries.

K. pneumoniae and P. mirabilis population structure.

The similarities detected between *K. pneumoniae* and *P. mirabilis* from companion animals and humans points to its possible zoonotic nature. Futures studies using WGS are needed to ultimately link the population structure and molecular antimicrobial resistance epidemiology of *K. pneumoniae* and *P. mirabilis* of both origins. Furthermore, *K. pneumoniae* and *P. mirabilis* from other types of infection, besides UTI, need to also be compared in the future to investigate additional similarities and risk factors.

The development of a MLST scheme for *P. mirabilis* typing would improve the comparability of results worldwide while WGS techniques becomes more affordable and user-friendly.

Transmission dynamics of K. pneumoniae and P. mirabilis between companion animals and humans.

After demonstrating that healthy dogs and humans may share *K. pneumoniae* and *P. mirabilis* strains, new studies using a longitudinal design are needed to clarify the colonisation length and transmission dynamics over time. The use of a larger sample size would also allow to identify possible risk factors and transmission routes leading to the detection of potential control measures that would limit the dissemination of resistant and/or virulent *K. pneumoniae* and *P. mirabilis*. Studies on a higher number of cats should also be conducted.

Future colonisation studies should also be conducted in households with infected companion animals and/or humans to evaluate if infection (UTI or other) increases the chances of *K. pneumoniae* and *P. mirabilis* sharing.

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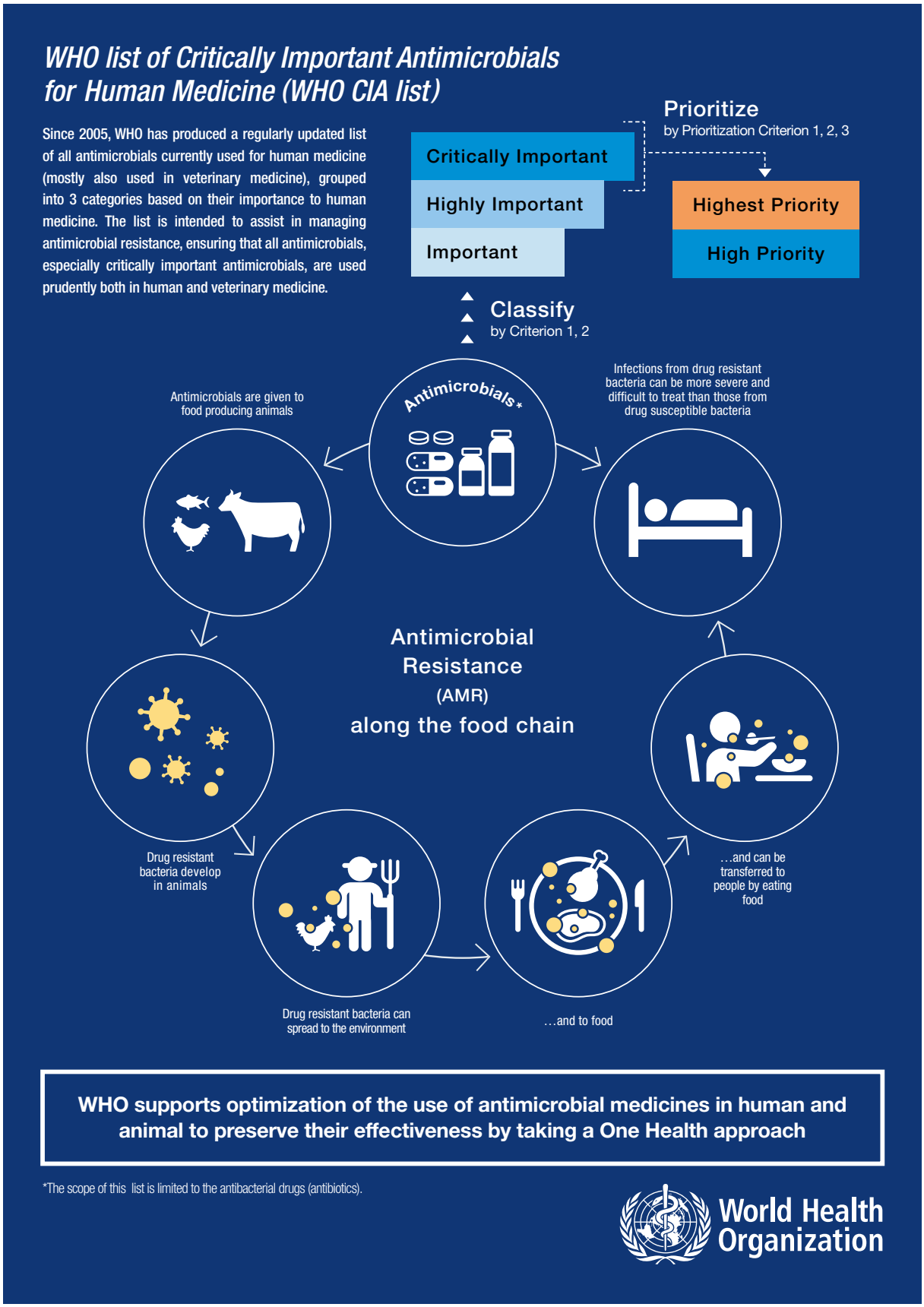
Annex 1 - WHO list of critically important antimicrobials for human medicine (WHO CIA list)



WHO Critically Important Antimicrobials for Human Medicine 5th revision

Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR)

October 2016

Summary of classification and prioritization of antimicrobials categorized as Critically Important, Highly Important and Important

Medically Important Antimicrobials

Antimicrobial class	Criterion (Yes = ●)				
CRITICALLY IMPORTANT ANTIMICROBIALS	C1	C2	P1	P2	P3
HIGHEST PRIORITY					
Highest Priority — *Cephalosporins (3rd, 4th and 5th generation)*	●	●	●	●	●
Highest Priority — *Glycopeptides*	●	●	●	●	●
Highest Priority — *Macrolides and ketolides*	●	●	●	●	●
Highest Priority — *Polymyxins*	●	●	●	●	●
Highest Priority — *Quinolones*	●	●	●	●	●
HIGH PRIORITY					
Aminoglycosides	●	●		●	●
Ansamycins	●	●	●	●	
Carbapenems and other penems	●	●	●	●	
Glycylcyclines	●	●	●		
Lipopeptides	●	●	●		
Monobactams	●	●	●		
Oxazolidinones	●	●	●		
Penicillins (natural, aminopenicillins, and antipseudomonal)	●	●		●	●
Phosphonic acid derivatives	●	●	●	●	
Drugs used solely to treat tuberculosis or other mycobacterial diseases	●	●	●	●	

Critically Important

HIGHLY IMPORTANT ANTIMICROBIALS	C1	C2	P1	P2	P3
Amidinopenicillins		●			
Amphenicols		●			
Cephalosporins (1st and 2nd generation) and cephamycins		●			
Lincosamides		●			
Penicillins (anti-staphylococcal)		●			
Pseudomonic acids		●			
Riminofenazines	●			NA	
Steroid antibacterials		●			
Streptogramins		●			
Sulfonamides, dihydrofolate reductase inhibitors and combinations		●			
Sulfones	●				
Tetracyclines	●				

Highly Important

IMPORTANT ANTIMICROBIALS	C1	C2	P1	P2	P3
Aminocyclitols					
Cyclic polypeptides					
Nitrofurantoins				NA	
Nitroimidazoles					
Pleuromutilins					

Important

C1 Criterion 1

The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people.

C2 Criterion 2

The antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from nonhuman sources, or (2) bacteria that may acquire resistance genes from nonhuman sources.

P1 Prioritization criterion 1

High absolute number of people, or high proportion of use in patients with serious infections in health care settings affected by bacterial diseases for which the antimicrobial class is the sole or one of few alternatives to treat serious infections in humans.

P2 Prioritization criterion 2

High frequency of use of the antimicrobial class for any indication in human medicine, or else high proportion of use in patients with serious infections in health care settings, since use may favour selection of resistance in both settings.

P3 Prioritization criterion 3

The antimicrobial class is used to treat infections in people for which there is evidence of transmission of resistant bacteria or resistance genes from non-human sources.

WHO CIA list 5th rev. : http://who.int/foodsafety/publications/antimicrobials-fifth/en/

AGISAR: http://who.int/foodsafety/areas_work/antimicrobial-resistance/agisar/en

WHO/NMH/FOS/FZD/17.1

World Health Organization

http://www.who.int/foodsafety/publications/cia2017.pdf?ua=1