UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

USE OF ANTIMICROBIALS AND CEPHAMICIN RESISTANCE IN COMPANION ANIMALS

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINARIA

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I dedicate this work to João and my family for making this possible….

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To my family, for believing in me

Abstract

USE OF ANTIMICROBIALS AND CEPHAMICIN RESISTANCE IN COMPANION ANIMALS

Objectives: This work includes two separate studies. In study 1 the aim was to investigate the use of antimicrobials in companion animals in Portugal while in study 2 the objective was to evaluate and characterize the prevalence of *bla*$_{CMY-2}$ gene in Enterobacteriaceae and the phylogenetic relatedness among plasmids from companion animals and humans.

Materials and Methods: In study 1 in order to understand the patterns of antimicrobial prescription a national survey was submitted to veterinarians. In study 2 plasmids harboring *bla*$_{CMY-2}$ were transferred into GeneHog® *E. coli* by electroporation and typed by S1 endonuclease pulsed-field gel electrophoresis, PCR-based replicon typing, and plasmid multilocus sequence typing (pMLST).

Results: In study 1, the use of amoxicillin-clavulanate (28%) and enrofloxacin (18%) were the most common antimicrobials used in dogs and cats, whereas clindamycin (3%) cefovecin (2%) and pradofloxacin (2%) were the less prescribed. In study 2, twenty three *bla*$_{CMY-2}$ genes were plasmid encoded. Replicon typing demonstrated that from animal isolates, thirteen isolates were IncFII plasmids, five isolates were IncI1 plasmid, one isolate carried an A/C plasmid and the remaining isolate was non-typeable by PBRT. Regarding human isolates, one isolate was IncFII, one was IncI1 and the third isolate was also non-typeable. IncI1 *bla*$_{CMY-2}$ plasmids showed that three were sequence type (ST2), three were non-typeable and fourteen IncFII plasmids were F2;FIA-;FIB- by pMLST.

Conclusions and Clinical Importance: This work showed that in order to understand how antimicrobials are prescribed, further studies and implementation of a surveillance system for antimicrobial usage in these species would be recommended. Plasmid encoded resistant genes are an important factor for selection and dissemination of genes such as *bla*$_{CMY-2.}$ The transmission of resistant genes in humans and animals is due to plasmid encoding which is of great concern, and further research is still necessary to understand about the mechanisms which have led to the rapid spread of resistant bacteria worldwide.

Keywords: antimicrobials, *bla*$_{CMY-2}$ gene, plasmid

Resumo

USO DE ANTIMICROBIANOS E RESISTÊNCIA ÀS CEFAMICINAS EM ANIMAIS DE COMPANHIA

Objetivos: Este trabalho inclui 2 estudos. O objetivo do primeiro estudo consistiu em investigar o uso de antimicrobianos em animais de companhia em Portugal enquanto no segundo estudo, o objetivo consistiu na análise e caraterização da prevalência do gene *bla*$_{CMY-2}$ em Enterobacteriaceas, ao mesmo tempo que pretendeu determinar a semelhança filogenética dos respetivos plasmídeos, em animais e humanos.

Materiais e Métodos: No estudo 1, para compreender os hábitos de prescrição de antimicrobianos em Portugal foi realizado um inquérito nacional aos Veterinários. No estudo 2, os plasmídeos com o gene *bla*$_{CMY-2}$ foram transferidos para uma célula electrocompetente GeneHog® *E. coli* por electroporação, e caraterizados por S1 endonuclease pulsed-field gel electrophoresis, PCR-based replicon typing e plasmid multilocus sequence typing (pMLST).

Resultados: No estudo 1, os antimicrobianos mais utilizados em cães e gatos foram a amoxicilina/acido clavulânico (28%) e enrofloxacina (18%). Clindamicina (3%), cefovecina (2%) e pradofloxacina (2%) foram os menos utilizados em ambas as espécies. No estudo 2, vinte e três genes *bla*$_{CMY-2}$ estavam codificados em plasmídeos. De acordo com o método replicon typing, os isolados de origem animal, treze pertenciam ao plasmídeo IncFII, cinco estavam codificados no plasmídeo IncI1, um estava presente no plasmídeo A/C e um isolado foi considerado “non-typeable”. Dos 3 isolados humanos, 1 estava incorporado num plasmídeo IncI1, 1 estava inserido no plasmídeo IncFII e o terceiro foi considerado “non-typeable”. Pelo método pMLST, os plasmídeos IncI1 foram caraterizados como ST2, e três foram considerados “non-typeable”. Catorze plasmídeos IncFII foram caraterizados como sendo F2;FIA-;FIB-.

Conclusões e Importância Clínica: Para compreender os hábitos de prescrição de antimicrobianos, seriam recomendáveis estudos complementares e a implementação de um sistema de monitorização para o consumo de antimicrobianos nestas espécies. A presença de genes de resistência em plasmídeos é um fator importante para a seleção e disseminação de genes como o gene *bla*$_{CMY-2}$. A transmissão destes genes em humanos e animais é mediada por plasmídeos, o que é preocupante. Investigação contínua é pois necessária para entender quais os principais mecanismos que conduziram à disseminação de bactérias com genes de resistência no mundo.

Palavra-chave: antimicrobianos, gene, *bla*$_{CMY-2}$, plasmídeo

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List of abbreviations and symbols

APIFARMA	Associação Portuguesa da Indústria Farmacêutica
AVMA	American Veterinary Medical Association
Bp	Base pairs
BHIB	Brain Heart Infusion Broth
BSAVA	British Small Animal Veterinary Association
CLSI	Clinical Laboratories Standards Institute
SvHKS	Danish Small Animal Veterinary Association
DNA	Deoxyribonucleic Acid
DGAV	Direcção Geral de Alimentação e Veterinária
dNTPs	Deoxyribonucleotide Triphosphates
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EMA	European Medicines Agency
e.g.	Exempli gratia
ESAC	European Surveillance Antimicrobial Consume
ESBL	Extended Spectrum β-Lactamases
ESVAC	European Surveillance Veterinary Antimicrobial Consume
EHEC	Entero haemorrhagic Escherichia coli
ETEC	Enterotoxigenic Escherichia coli
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility testing
FLUTD	Feline Lower Urinary Tract Disorders
IS	Inserted Sequences
MARAN	Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands
MBL	Metallo β-lactamases
MIC	Minimum Inhibitory Concentration
OMV	Ordem dos Médicos Veterinários
OIE	World Organization for Animal Health
pAmpC	Plasmid AmpC β-lactamases
pMLST	Plasmid Multilocus Sequence Typing
PBRT	PCR-based Replicon Typing
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
R	Resistant
S	Susceptible
SVA	Swedish Veterinary Association
SWEDRES-SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring
ST	Sequence Type
TBE	Tris-borate-EDTA
UTI	Urinary Tract Infection
VMD	Veterinary Medicines Directorate
WHO	World Health Organization
µL	Microliter

1. Introduction

During the World War II the production and use of penicillin was implemented for the first time as a response to treat the war causalities. In the last stage of this war the lyophilized preparations of penicillin were made available for veterinarians who reconstituted the antibiotic with saline for intramammary infusions to treat bovine mastitis. This fact represented a significant advance because penicillin proved to be more effective than treatments previously available for dairy animals. The variety of antibiotics, the routes of administration and the reasons for their use expanded during the period between 1950 and 1960 (Gustafson & Bowen, 1997).

Antimicrobials are medicines that kill or inactivate microbes. They include antibiotics, which are used against bacteria. After being exposed to an antimicrobial repeatedly, microbes can undergo changes that stop them being killed or inactivated by the treatments. This is known as antimicrobial resistance and is a growing problem in human and veterinary medicine. During the last decade a number of events have concerned the scientific community and increased the awareness of public health issues related to antimicrobial resistance. For example, the emergence of extended spectrum β-lactamases (ESBLs) it is a concern to human health due to the possibility of transfer of resistance genes across bacteria populations. The risks for humans related to antimicrobial resistance in animals are not circumscribed to foodborne risks only (European Medicine Agency [EMA], 2009a). When the microorganisms become resistant to most antimicrobials they are often referred to as "superbugs". One the issue is that bacteria become resistant to critically important antimicrobials (World health Organization [WHO], 2011). To address the need for universal terms for the various degrees of antimicrobial resistance, the following definitions were suggested. A pandrug resistant (PDR) isolate is designated as resistant to all available classes of antimicrobials, while the definition of extensive drug resistance (XDR) refers to a pathogen which is resistant to all but one or two classes. A strain is considered a multidrug resistant (MDR), if an isolate is resistant to three or more classes of antibiotics (β-lactams, aminoglycosides, fluoroquinolones, sulfonamides, and tetracyclines) (Falagas & Karageorgopoulos, 2008).

The impact of antimicrobial use and possible misuse in animals as a public health issue is focused on food-producing animals mainly. However there are not many studies about the use of antibiotics in companion animals. The direct contact with pets makes it possible for the transference of resistant genes between companion animals and humans to occur. It is necessary to control and monitor the antimicrobial use in animals in order to avoid the increase of antimicrobial resistance. The European commission established an "Action plan against the rising threats from antimicrobial resistance" released in November 2011 to

promote antimicrobial surveillance in the different sectors involved actions regarding research and developing of novel antimicrobials (European Commission, 2011). The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project was started in 2010 by the European Medicines Agency (EMA). The aim of the project is to collect information every year on the use of antimicrobials agents from Europeans state members. This information is considered critical in order to monitor and identify risk factors associated with antimicrobial usage in animals.

The awareness of this problem, together with the increase of antimicrobial resistance among companion animals and the interest by the author in this area led to this research project. The author aims to determine and characterize the resistance in Enterobacteriaceae to cephamycin in order to contribute to a better understanding of the mechanisms of development of antibiotic resistance. By understanding these mechanisms it can be possible to undertake new strategies to fight antibiotic resistance. The author also evaluates the use of antimicrobial agents in companion animals in Portugal through a national survey which was online during the time of the training period. The first part of this document will include a literature review of the main topics. The second part refers to the studies developed including its main goals, materials and methods, results, discussion and concluding remarks of this study. The terms antimicrobial and antibiotic, Rep type and Inc group to describe plasmids are used interchangeably in this thesis.

1.1. Training period

To accomplish the project, the author took a training period of approximately six months in two different locations. The first place was at the Laboratory of Antimicrobial Resistance and Biocides, Faculty of Veterinary Medicine, University of Lisbon, Portugal (FVM-UL) from September 20th to December 15th 2012, the remaining period was undertaken at LLP/Erasmus Program in the Microbiology Laboratory of the Faculty of Life Sciences, University of Copenhagen (KU-SUND) from January 10th to April 30th 2013, under the supervision of Professor Luca Guardabassi and Dr. Valeria Bortolaia.

Part 1

Literature Review

2. Literature Review

2.1. The family Enterobacteriaceae

2.1.1 Typical characteristics

The family Enterobacteriaceae is considered to be the most important family which can cause a variety of community and nosocomial infections (meaning that are acquired in hospital environment) in humans such as septicaemia, urinary tract infections (UTI), pneumonia, cholecystitis, cholangitis, peritonitis, wound infections, meningitis, and gastroenteritis. Enterobacteriaceae falls within the domain Bacteria, phylum *Proteobacteria*, class Gammaproteobacteria, and order *Enterobacteriales*. In Table 1 are listed the most common genera and species belonging to this family. The members of this family are gram-negative, rod-shaped, non-spore-forming facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase but do not produce oxidase (Donnenberg, 2010).

2.1.2. Natural habitat

Most Enterobacteriaceae are designated as enteric because the main habitat of most of them is the lower gastrointestinal tract of animals and humans, but the environment can also be a reservoir (Farmer, Boatwright & Janda, 2008). In large intestine there is a complex and dynamic interaction with high densities of living bacteria, which achieve concentrations of up to 10^{11} or 10^{12} cells/g in the luminal contents (Guarner & Malagelada, 2003).

Table 1 - Important genera and species of the family Enterobacteriaceae and most common type of infections (adapted from Abbott, 2008; Farmer *et al*., 2008; Nataro, Bopp, Fields, Kaper & Stockbine, 2008; Donnenberg, 2010).

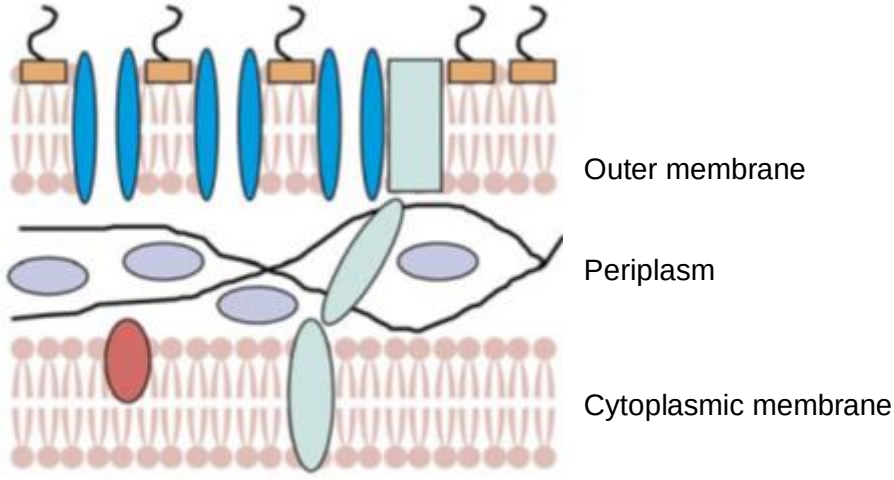
Genus	Species	Common type of infections
Citrobacter	*Freundii*	UTIs, pneumonia, meningitis, septicaemia, wound infections
Enterobacter	*cloacae, aerogenes*	UTIs, pneumonia, septicaemia, wound infections
Escherichia	*Coli*	UTIs, diarrhoea, septicaemia, meningitis
Klebsiella	*pneumonia, oxytoca*	UTIs, pneumonia, septicaemia
Morganella	*Morganii*	UTIs, septicaemia
Proteus	*mirabilis, vulgaris*	UTIs, pneumonia, septicaemia, meningitis, wound infections
Salmonella	*Enterica*	diarrhoea, typhoid fever, septicaemia, UTIs, osteomyelitis
Serratia	*Marsecens*	UTIs, pneumonia, wound infections, septicaemia
Shigella	*Dysenterii*	diarrhoea, dysentery
Yersinia	*pestis, enterocolitica*	plague, enteritis, diarrhoea, septicaemia

2.1.3. Cell wall structure

The structure from gram-negative bacteria is different from gram-positive; gram-negative bacteria cell wall is composed of a thin peptidoglycan layer, also named as murein (Beveridge & Graham, 1991; Donnenberg, 2010). The murein consists of alternating N-acetylglucosamine and N-acetylmuramic acid amino sugars joined by β-1,4 linkages, with a short peptide composed of L-alanine, D-glutamic acid, L-meso-diaminopalmelic acid, and D-alanine attached to the carboxyl group of the muramic acid (Donnenberg, 2010).

The periplasmatic space ranges from 20%- 40% of total cell volume in a gram-negative bacteria, and is considered an integral compartment of the gram-negative cell wall (Stock, Rauch & Roseman, 1977; Beveridge, 1999). Together the plasma membrane and the cell wall (outer membrane, peptidoglycan layer, and periplasm) constitute the gram-negative envelope (Beveridge & Graham, 1991). The envelope is responsible for maintaining the shape and osmotic stability of the cell; however in the replication and elongation process of the bacteria is always being remodelled (Figure 1) (Beveridge & Graham, 1991).

Figure 1 - General structure of Enterobacteriaceae (reproduced with permission from Livermore & Woodford, 2006)



2.2. Principals genera from Enterobacteriaceae family

2.2.1. *Escherichia coli*

Escherichia coli are the most important bacteria in the normal microbiota of humans and animals. It colonizes the gastro intestinal tract within hours after birth and there is a mutual relationship (symbiotic) between host and bacteria. However if the immune system or if the gastrointestinal barriers are compromised some strains can cause disease in gastro intestinal tract or urinary system for example (Nataro & Kaper, 1998). *E. coli and Klebsiella pneumonia* are among the most important causes of serious hospital-acquired and

community-onset bacterial infections in humans (Paterson, 2006). The regular presence of *E. coli* in the human intestine and faeces has led to tracking the bacterium in nature as an indicator of faecal pollution and water contamination (Goyanes *et al*., 2007; Todar, 2008).

2.2.2. *Proteus* spp.

The two species most representative of this genus are: *P. mirabilis* and *P*. *vulgaris* which were both described in 1885 by Hauser. He noted the swarming colonies nature and classified them according to their ability to liquefy gelatine (O'Hara, Brenner & Miller, 2000). This swarming ability is related to the flagella which translocate very fast along the plates, and this characteristic allows then to differentiate from other Enterobacteriaceae. The genus *Proteus* spp*.* are lactose negative and motile (Donnenberg, 2010).

Bacteria of the genus *Proteus* are a part of the commensal flora of the intestinal tract from humans and animals (O'Hara *et al*., 2000; Giammanco, Pignato, Grimont, Grimont & Giammanco, 2011) . *P. mirabilis* and *P. vulgaris* account for the majority of clinical isolates from this genus, acting also as opportunistic pathogens causing primary and secondary infections (Donnenberg, 2010; Giammanco *et al*., 2011).

2.2.3. *Klebsiella* spp.

The genus *Klebsiella* was named by Trevisian in 1885 to honour microbiologist Edwin Klebs (Brisse, Grimont & Grimont, 2006). *Klebsiella* spp. is ubiquitous in nature, can be found in the environment (water, soil, and plants) but also in the mucosa from humans and animals. *Klebsiella pneumoniae* is the most important species of the genus and is responsible together with *Klebsiella oxytoca* for 8% nosocomial bacterial infections in Europe (Podschun & Ullmann, 1998).

2.2.4. *Enterobacter* spp.

The genus *Enterobacter* includes 14 species, *Enterobacter aerogenes* and *Enterobacter cloacae* are by far the most important and relevant pathogens in humans among the genus *Enterobacter. Enterobacter* spp. is considered an opportunistic pathogen, and recently has become an important cause of nosocomial infections (Sanders & Sanders, 1997).

2.2.5. Other Genus

Other important Enterobacteriaceae species are: *Salmonella* spp*.*, *Citrobacter* spp., *Shigella* spp. and *Serratia* spp. Salmonella is a facultative intracellular pathogen acquired by consumption of water and/or contaminated food. In contrast to other pathogens *Salmonella* spp. requires a large number of genes to become virulent (Groisman & Ochman, 1997). The genus *Salmonella* includes 10 species but only two are clinically important: *S. enterica*, which

is subdivided into over 2,000 serovars, and *Salmonella bongori*. The most important serovars of *S. enterica*, are: *S. typhi*, which is responsible for systemic infections and typhoid fever infecting only humans; and *S. typhimurium*, which is a leading cause of gastroenteritis in human and other mammalian species increasing the incidence of non-typhoid *Salmonella* infections worldwide (McClelland *et al*., 2001).

The genus *Citrobacter* spp. includes twelve species and is an important cause for opportunistic infections. *Citrobacter* species are commonly found in water, soil, food, and the intestinal tracts of animals and humans (Shih, Chen, Chang, Luh, & Hsieh, 1996; Donnenberg, 2010).

In 1940, four species of the new genus *Shigella* spp. were recognized by Boydii as the cause for dysenteric disease in humans. *Shigella* and *E. coli* have always been considered to be very closely related, but *E. coli* strains became a different genus due to differences in medical significance (Lan & Reeves, 2002).

Ten species are known to belong to *Serratia* genus and the most important species are: *S. marsecens* which can be differentiated from other bacteria by producing a red pigment named Prodigiosin; and *S. liquefascens*. These species can be found in water, mammals and also in hospitalized patients (Grimont & Grimont, 2006).

2.3. Clinical significance of Enterobacteriaceae

The family Enterobacteriaceae as referred previously is indeed the most important family which colonize the gastrointestinal tract since early age and coexist with animals and humans with mutual benefits for decades. In many situations the infections caused by these species are nosocomial. As it is shown in table 1, Enterobacterial strains are responsible for a variety of clinical situations such as urinary tract infection, pneumonia, and sepsis (Kaper, Nataro & Mobley, 2004).

The species *Escherichia coli* causes diverse intestinal and extraintestinal diseases by means of virulence factors, named serotypes. The main causes of diarrhoea in animals and humans is triggered by enterotoxigenic *E. coli* (ETEC) and entero haemorrhagic *E. coli* (EHEC) (Kaper *et al*., 2004). *E. coli* is also considered an important pathogen in canine UTIs (Feria, Ferreira, Correia, Gonçalves & Caniça, 2002).

Klebsiella spp. is an opportunistic ubiquitous pathogen and is a major cause of nosocomial infections. The targets are usually immune-compromised individuals that are hospitalized with chronic diseases. *Klebsiella pneumoniae* is responsible for most of the outbreaks in health care facilities. This species is also involved in urinary tract infection, pneumonia, septicaemia and soft tissue infections (Podschun & Ullmann, 1998; Struve & Krogfelt, 2004). In animals it can act as an opportunistic pathogen it has been implicated in cases of mastitis in cattle, metritis in mares, bacteraemia in calves, pneumonia and urinary tract infections in

dogs (Roberts, McClain, Hansen, Currin & Howerth, 2000). The importance of *Klebsiella* is based on the increasing reports of clinical situations caused by multiresistant strains capable of producing extended spectrum β- lactamases (Podschun & Ullmann, 1998).

Of the Yersinia genus three species *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis* are well known animal and human pathogens. Pathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis* cause yersiniosis, an acute enteric disease, in humans and animals (Stamm, Hailer, Depner, Kopp & Rau, 2013). Y. *enterocolitica* is regarded as a significant food-borne pathogen. Besides food producing animals, companion animals are also considered a reservoir for human *Yersinia* infections (Stamm *et al*., 2013). *Y. pestis* is responsible for the plague, a zoonotic disease transmitted to human through a vector (fleas) (Linde, Neubauer, Meyer, Aleksic & Lehn, 1999). In contrast to other Enterobacterea, *Yersinia pestis* circulates in the bloodstream, lymphatic vessels and organs like spleen and liver, and may contact with Enterobactereaceas when reaching the bloodstream. To date there have been 3 major plagues and a high number of casualties (Galimand, 1997).

The family Enterobacteriaceae is the most important and relevant in clinical therapy because it is a common source of infections in animals, community and hospitals spreading easily between humans (Pitout, 2008a). These bacteria also have the ability to acquire new genetic material through horizontal gene transfer (HGT), mediated by plasmids or transposons. This combination represents an important issue regarding the emergence of multidrug resistance in Enterobacteriaceae and lights up the possibility of interspecies transfer of resistance determinants (Wise *et al*., 1998; Nordmann, Dortet & Poirel, 2012).

2.4. Antimicrobials – The era of antimicrobials

"One sometimes finds what one is not looking for" - Alexander Fleming

The general term "antibiotic era" is usually associated with the names of Paul Ehrlich and Alexander Fleming. Ehrlich's wanted to find a drug against syphilis which targeted only the source of infection caused by the spirochete *Treponema palladium*. Syphilis was considered an endemic disease and almost incurable at the time. In 1909 after several trials with rabbits an effective compound was discovered, marketed under the name *Salvarsan*, and for many years was the most prescribed drug until its replacement by penicillin (Aminov, 2010).

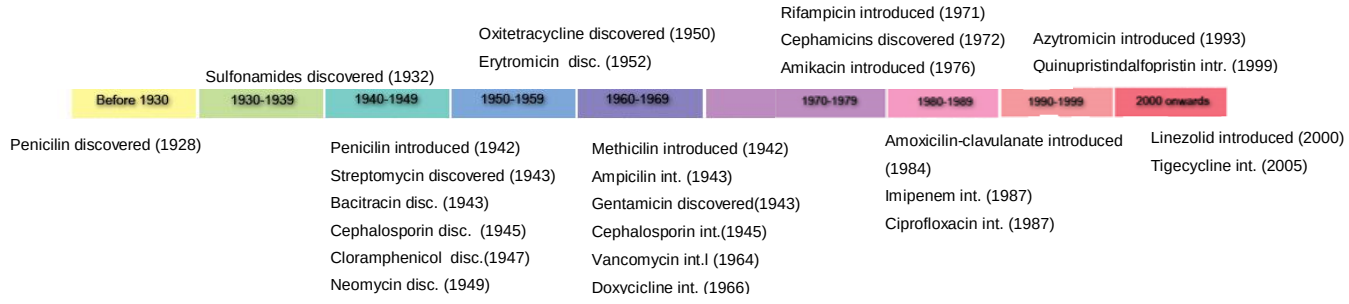
The introduction of antibiotics in the 1940s was considered as one of the greatest advances/achievements in therapeutic medicine for treating and preventing infectious diseases in human medicine. An antibiotic can be defined as a natural compound which is produced from fungi, bacteria and other microorganisms that can kill or inhibit the growth of bacteria, while the term antimicrobial refers to a group of substances, natural or synthetic which includes also the antibiotics, antifungals, antiprotozoals and antivirals (WHO, 2011). The antimicrobials can be classified as natural when they are produced by microorganisms,

or synthetic when the chemical scaffolds were used later to create new generations of clinically useful antibiotics by chemical modification (Peláez, 2006). The first antibiotic named penicillin was discovered by Alexander Fleming in 1928 when he observed that a common mould (*Penicillium notatum*) produced a substance that inhibited the growth of colonies of *Staphylococcus* spp. (Ligon, 2004).

Howard Florey and Ernst Chain, both part of an Oxford team, were the scientists who in 1940 were able to purify penicillin for clinical testing. The period from the 1950s throughout the 1970s is defined as the golden era of discovering new classes of antimicrobial agents (Aminov, 2010). Since year 1993 there has not been many new classes of antimicrobials developed by industry and that we might face a shortage of therapeutic options against multi-resistant bacteria. That is well supported by Figure 2.

Nowadays there is growing concern about the increase of antibiotic resistance increase worldwide. The use of antibiotics is considered to be the main factor for pathogenic bacteria growth which has led to the dissemination of resistant bacteria and resistant genes. This situation applies to both human and veterinary practice, since antimicrobials are used for similar purposes (e.g. prophylaxis therapy of infectious diseases) in humans and animals. This can lead to the increase of levels of resistance traits in pathogenic and commensal bacteria. Resistant bacteria can infect humans by direct contact or via food products of animal origin (Van den Bogaard & Stobberingh, 2000). So far many studies have emphasised mainly in food producing animals. To decrease the risk of transmission of antimicrobial residues and pathogens through the food chain some countries have developed and are focusing on monitoring antimicrobial usage in food-producing animals (Aarestrup & Wegener, 1999).

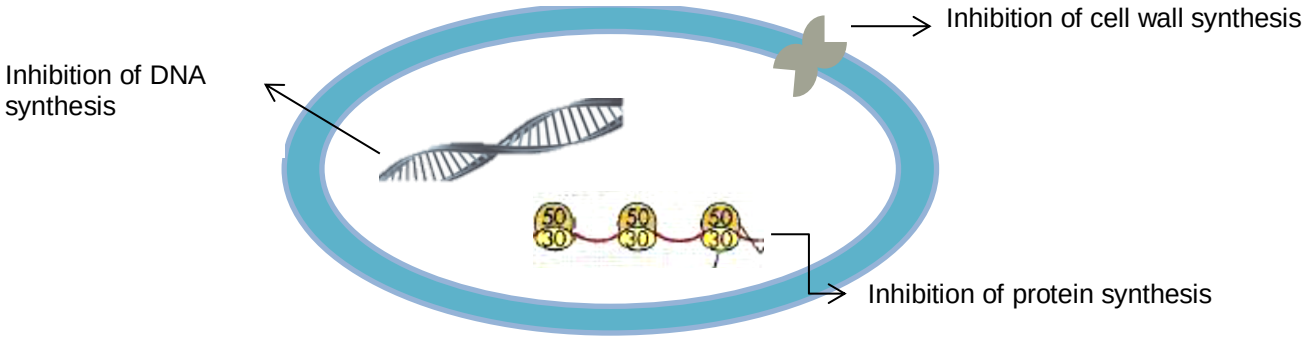
Figure 2 - Discovery of antimicrobials (adapted from http://amrls.cvm.msu.edu/pharmacology/historical-perspectives/the-golden-age-of-antibacterials).



2.4.1. Classification of antibacterial agents

Antibacterial agents can be classified according to their mechanism of action (how antimicrobial growth is supressed) and by spectrum of activity. The five main target sites of antimicrobial agents are: (i) cell wall synthesis, (ii) protein synthesis, (iii) nucleic acid synthesis, (iv) metabolic pathways, and (v) cell membrane functions (Figure 3) (Tenover, 2006; Maddison, 2009). Antibacterial drugs that act by inactivating the wall synthesis include the penicillins, cephalosporins and vancomycin. Aminoglycosides and tetracycline inhibit protein synthesis by binding to the ribosomal subunit 30S, while macrolides and chloramphenicol bind to the 50S ribosomal subunit to inhibit protein synthesis. Fluoroquinolones antimicrobial agents disrupt the DNA synthesis whereas sulphonamides and trimethoprims block the pathway for folic acid synthesis (Tenover, 2006).

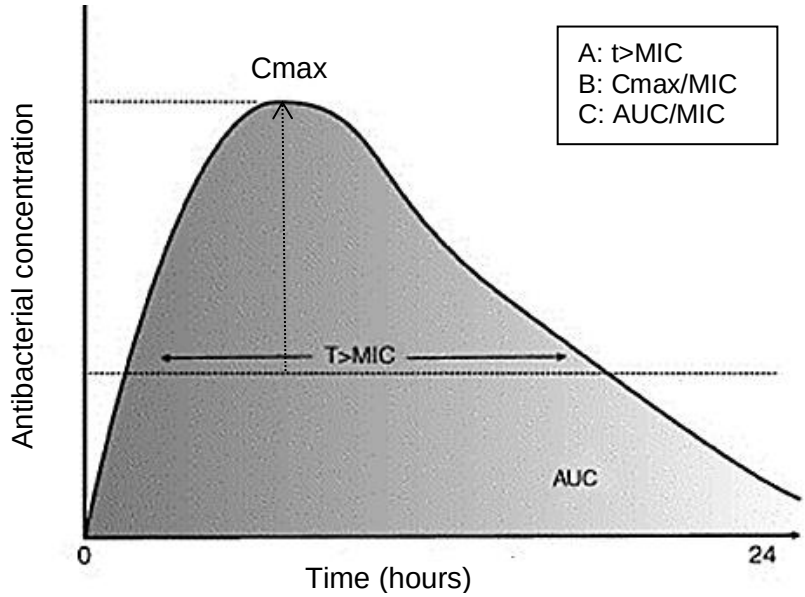
Figure 3 – Principal antimicrobial action mechanisms (Original illustration).



Antibacterial agents can be classified as bactericidal or bacteriostatic. This classification is used mainly for clinical purposes and is not consistent to all bacteria. The ability of an antimicrobial agent to inhibit or kill a microorganism is relative and depends on the bacteria. For example chloramphenicol inhibits the growth (bacteriostatic) of *E. coli* while it kills (bacteriocidal) *Haemophilus influenza* (Maddison, 2009). Bacteriostatic agents inhibit bacterial growth as long as the drug concentration is above the minimal inhibitory concentration (MIC). Tetracycline, chloramphenicol and sulphonamides are examples of bacteriostatic agents. Bactericidal agents e.g. aminoglycosides, cephalosporins, fluoroquinolones, metronidazole, penicillins and potentiated sulphonamides kill the bacteria and are preferred in infections in which the host cannot control or eradicate the infection due to location of the infection itself or due to the compromised immune status of the host. Bactericidal agents can also be further classified as time-dependent (penicillins and cephalosporins, both slow bactericidals), or concentration-dependent drugs. For bactericidal antibiotics, plasma levels of antibiotic concentration must be above the MIC as long as

possible (each 24 hours of treatment). In contrast, for concentration-dependent drugs (aminoglycosides and fluoroquinolones), the higher the plasma peak is achieved, the greater the proportion of bacteria killed. The peak concentrations, the area under the plasma concentration versus time curve are important for bactericidal success (Maddison, 2009). In order to a better understanding of antibiotic activity it is necessary to determine some important parameters as illustrated in Figure 4. The first parameter, time at which concentration is above the MIC (t > MIC), is related to bactericidal effects over time. It refers to the time in which the drug is above the MIC and is dependent on the half-life, dosage, frequency of administration of the drug over a certain time period. The parameter corresponding to the ratio peak plasma concentration (Cmax)/MIC, it is the maximum concentration of a certain drug in the plasma, relates bactericidal effects with concentration, and is mainly dependent on the unit dose and the volume of distribution of the drug. The last parameter, area under the concentration-time curve (AUC)/MIC, combines both types of effects, because it refers to the total amount of drug to which bacteria are exposed over the time period, and is directly related to the total dose given during that period and inversely proportional to the drug clearance (Bambeke, Barcia-Macay, Lemaire & Tulkens, 2006).

Figure 4 - Antibacterial concentration vs. time graph illustrating pharmacokinetic and pharmacodynamics parameters (adapted from Lister, 2006).



The effectiveness of individual drugs against the isolated organism is categorized according to the new ISO 20776-1 standard using the following categories: "Susceptible", “Intermediate” and “Resistant” depending on the MIC value. The MIC is defined as the minimum concentration of an antibiotic that is able to prevent the further growth of the infectious organism *in vitro* (Rodloff, Bauer, Ewig, Kujath & Müller, 2008). Susceptible (S), a bacterial strain is said to be susceptible to a given antibiotic when it is inhibited in vitro by a

concentration of this drug that is associated with a high rate of therapeutic success. Intermediate (I), the sensitivity of a bacterial strain to a given antibiotic is said to be intermediate when it is inhibited in vitro by a concentration of this drug that is associated with an uncertain therapeutic effect. Resistant (R), a bacterial strain is said to be resistant to a given antibiotic when it is inhibited *in vitro* by a concentration of this drug that is associated with a high possibility of therapeutic failure. The European Committee on Antimicrobial Susceptibility testing (EUCAST) in order to harmonise and define breakpoints provides clinical breakpoints and epidemiological cut-off values (ECOFF). The ECOFF are used in clinical breakpoint development and as a sensitive indicator of resistance development in surveillance studies (European Committee on Antimicrobial Susceptibility testing [EUCAST], 2007).

2.5. β-lactam antibiotics

2.5.1. General structure and functions

The β-lactam bactericidal antibiotics, including – penicillins, cephalosporins, monobactams and carbapenems are one of the most widely used group of antimicrobials due do their safety, low cost, ease of delivery, minimal side effects and high efficacy. β-lactam antibiotics are grouped together based upon a shared structural feature (Wilke, Lovering & Strynadka, 2005). β-lactam is a generic name for all β-lactam antibiotics that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom (Wilke *et al*., 2005).

This group represents 60% of all antimicrobial used by weight and is used to treat infections caused by gram-negative bacteria and gram-positive, in human medicine (Livermore & Woodford, 2006). In veterinary medicine, different substances from the penicillin family, first to fourth generation cephalosporins and β-lactamase inhibitors are recommended for the treatment of companion animals according to the animal species involved and the underlying disease (Guardabassi, Jensen & Kruse, 2008; Smet *et al*., 2010).

2.5.2. Mechanism of action

β-lactam antibiotics inhibit the growth of bacteria by inactivating enzymes called penicillin-binding proteins (PBPs), located in the bacterial cell wall which are involved in the third stage of cell wall synthesis (Poole, 2004). β-lactam antibiotics normally interfere with this process by reacting covalently with the active site serine to form a stable acyl-enzyme preventing the peptidoglycan synthesis (Hujer *et al*., 2005). In contrast to gram-positive bacteria, in gram-negative the peptidoglycan it is a thin layer between the cell wall and the cytoplasmic membrane. The antibiotic β-lactam must diffuse across the outer membrane of the gram-negative cell, using pores formed by porin proteins, and then cross the periplasm before

reaching its PBP targets, which lie on the outer surface of the cytoplasmic membrane (Livermore & Woodford, 2006).

2.5.3. Penicillin

Penicillin was discovered in 1928 by Alexander Fleming and has activity against gram-positive and gram-negative bacteria (Tipper & Strominger, 1965). This class is considered the best known and first β- lactam antibiotic. All penicillin have a β-lactam, a thiazolidine ring and a side chain R group which is distinguishable between penicillins (Figure 5) (Baldo, 1999). In 1940 a large amount of penicillin was produced from cultures of *Penicillium notatum*. Several years after the first penicillin, penicillin G became available; however, it had some limitation such as instability in gastrointestinal (GI) and was not very effective against important gram-negative bacteria (Chambers, 2010).

2.5.4 Cephalosporins

This first isolation of *Cephalosporium acremonium* culture was discovered in 1948 by Giuseppe Brotzw but only became commercially available in 1962 (Botana, Landoni & Martín-Jiménez, 2002). Researchers at the University of Oxford isolated cephalosporin C in 1956 (Abraham & Newton, 1961). Some years after four classes of cephalosporins were produced (Botana *et al*., 2002). Cephalosporins of first generation have activity against gram-positive bacteria such as *Streptococcus, Corynebacterium, S. aureus e S. pseudintermedius* and limited activity towards gram-negative bacteria, as it shown in Table 2. *Enterococcus* spp*., Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) are resistant to these antibiotics. Cephalosporins are grouped into generations according to their spectrum of activity against gram-negative bacteria (Donowitz & Mandell, 1988). The cephamycins differ from the true cephalosporin by the presence of a methoxy-group in the position 7 of the cephalosporin group but are grouped together and classified according to their *in vitro* spectrum activity and structural similarities. Cephamicins remain stable to many β-lactamases (EMA, 2009b). In this document, the term “cephalosporin” will be used indiscriminately.

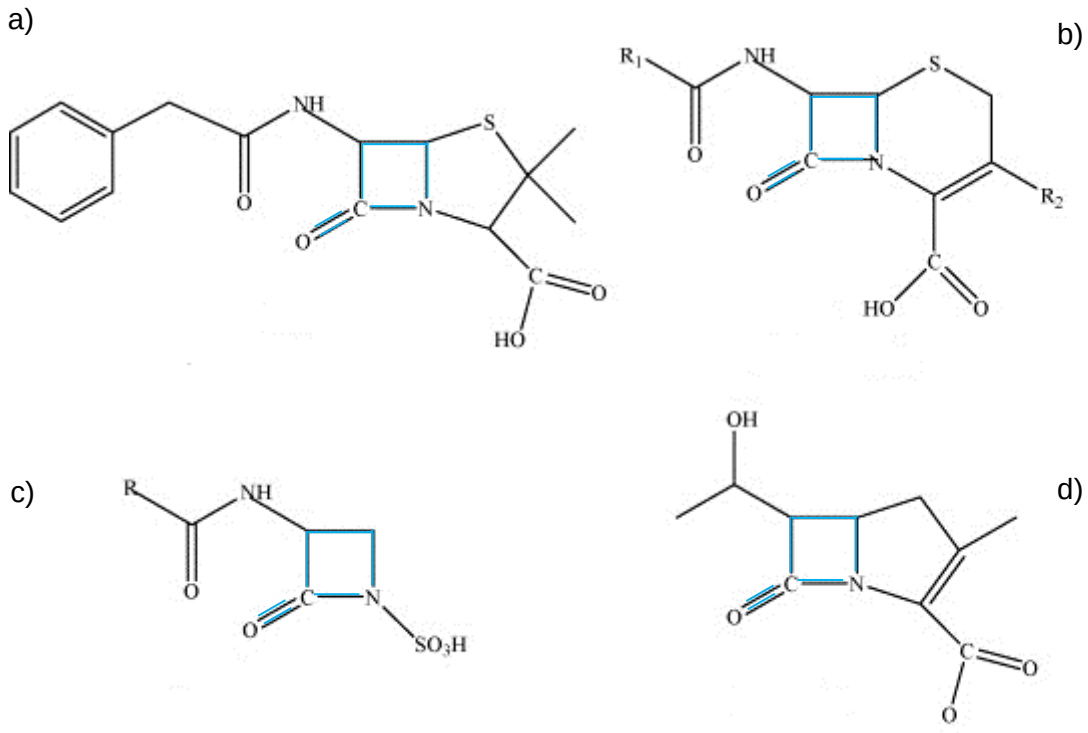
2.5.5 Monobactams

Monobactam antibiotics are a new class of β-lactam antibiotics. In contrast to penicillins or cephalosporins, monobactams have a monocyclic β-lactam structure and are produced by a range of bacterial species. Although these antibiotics exhibit poor antibacterial activity, they are highly stable to the action of β-lactamases produced by gram-negative bacteria (Sykes, Bonner, Bush, Georgopapadakou & Wells 1981; Saxon, Hassner, Swabb, Wheeler & Adkinson, 1984).

2.5.6. Carbapenems

Carbapenems are the class of β-lactam antibiotics with the broadest spectrum of activity (Nordmann *et al*., 2012). The activity includes many gram-positive, gram-negative and anaerobic bacteria; and no activity is recognized towards *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* and *Stenotrophomonas maltophilia*. The increase of β-lactamase resistance among Enterobacteriaceae led to the use of carbapenems widely, because they are stable to most β-lactamases including AmpC β-lactamases (Zhanel *et al*., 2007). Carbapenems are a last resource against bacterial infections used mainly when the infection is caused by a multidrug resistant pathogen (Bush, 2010).

Figure 5 - Different structure of b lactam antibiotics: a) penicilin, b) cephalosporin, c) monobactam, d) carbapenems. The β-lactam ring is shown in blue (reproduced with permission from Babic, Hujer & Bonomo, 2006).



2.5.7. β-lactamase inhibitors

The β-lactamase inhibition can be classified as either reversible or irreversible. Reversible inhibitors bind to an enzyme but activity may be restored after their removal. Irreversible inhibitors may be more effective than reversible inhibitors because the final outcome is the destruction of enzymatic activity. Specific irreversible inhibitors of β-lactamases include the clavulanic acid, the penicillanic acid sulfones sulbactam and aztreonam (Bush, 1988).

These substances are used in the treatment of serious Enterobacteriaceae and penicillin-resistant staphylococcal infections (Drawz & Bonomo, 2010). New compounds such as clavulanic acid and sulbactam exhibit low bactericidal activity when used alone but remain

effective against β-lactamases enhancing the activity of β-lactam antibiotics, hence the name (Moosdeen, Williams & Yamabe, 1988). Clavulanic acid was isolated from *Streptomyces clavuligerus* and was the first β-lactamase inhibitor introduced worldwide. Clavulanate (the acid from the solution) had weak bactericidal activity but combined with β-lactam antibiotics decreases the MIC values of some resistant bacteria (Reading & Cole, 1977). Later penicillinate sulfones, e.g. sulbactam and tazobactam were introduced in the clinical practice. These 3 substances are effective against many susceptible organisms expressing class A β-lactamases, which are the most common (including CTX-M and the ESBL derivatives of TEM-1, TEM-2, and SHV-1); and are generally less effective against class B, C, and D β-lactamases (Bush, 1988; Buynak, 2006). The combination of amoxicillin with clavulanate was selected due to the similarity in pharmacokinetics between the two compounds. Any new β-lactamase inhibitors should have the following characteristics: the molecules must be capable of preventing hydrolysis of well-tolerated broad-spectrum β-lactam antibiotic, preferably inexpensive penicillin; the pharmacokinetics of the two molecules should be similar; side effects should be minimal and mild; the molecules should not be good inducers of cephalosporinase activity. Ideally, one would like to have molecules with oral activity (Bush, 1988).

The use of β-lactamase inhibitors remains effective in the empirical treatment of respiratory, intra-abdominal, skin and soft tissue infections in humans. The use of β-lactamase inhibitors instead of cephalosporins appears to reduce the emergence of resistance in pathogens. In Portugal, amoxicillin-clavulanate is the first line antimicrobial for UTI infection in dogs, which leads to a strong selection pressure for the emergence of resistant bacteria (Feria *et al*., 2002). Similarly, their use may also curtail the emergence of other resistant pathogens such as *Clostridium difficile* and vancomycin-resistant enterococci (Lee, Yuen & Kumana, 2003).

Table 2 - Spectrum of β-lactam antibiotics within their use in Veterinary and Human Medicine -: no activity, (+): limited activity, +: active (adapted from Guardabassi *et al*., 2008; Hammerum & Heuer, 2009).

β-lactams	**Spectrum of activity**		**Use in Veterinary Medicine**	**Use in Human Medicine**
	gram-negative	gram-positive		
Penicillin	-	+	ampicillin, amoxicillin, benzylpenicillin	penicillin, ampicillin, amoxicillin
First generation cephalosporins	(+)	+	cephadroxil, cefapirin, cephalexin	cefalozin
Second generation cephalosporins	+	+	cefaclor, cefamandole, cefuroxime	cefuroxime, cefoxitin
Third generation cephalosporins	+	+	cefovecin, cefpodoxim, ceftiofur	ceftriaxone, cefotaxime, ceftazidime
Fourth generation cephalosporins	+	+	cefquinome	cefepime, cefpirome
Monobactams	+	-	not in use	aztreonam
Carbapenems	+	+	imipenem, meropenem	imipenem, meropenem, eartpenem

2.6. Antimicrobial resistance

Resistance to an antibiotic typically develops from its use according to Darwin´s principle: "survival of the fittest" (WHO, 2011). Antimicrobial resistance remains a global health concern and most countries have developed strategies to evaluate the level of resistance (Martins da Costa, Loureiro & Matos, 2013). This issue has emerged as a consequence of the selective pressure exerted by the overuse and misuse of antimicrobials in veterinary and human medicine. Furthermore, morbidity and mortality have increased in human medicine associated with resistant pathogens and thus failure therapeutic (Davies & Davies, 2010).

Recently, the World Health Organization (WHO) published a report establishing the critically important antimicrobial for human medicine (annex 1). The problematic of antimicrobial resistance becomes crucial when pathogens become resistant to these antimicrobials. The criteria for inclusion were: (1) sole therapy or one of few alternatives to treat serious human disease and (2) antimicrobial agents used to treat diseases caused by organisms that may be transmitted via non-human sources or diseases caused by organisms that may acquire resistance genes from non-human sources (WHO, 2007). Humans can acquire resistant pathogens or resistant genes of animal original directly from food consumption, direct contact with animals or through the environment (Rolain, 2013). In addition the use of the critically important antimicrobials should be minimised in animals to maintain/preserve the efficacy of these for treating human's infections (Danish Small Animal Veterinary Association [SvHKS], 2013).

2.6.1. Use of antimicrobials in food-producing animals

The introduction of antimicrobials in food-producing animals started in the 1940s to treat diseases and for growth purposes (Hammerum & Heuer, 2009).

In 1951 it was first reported in California the effects of using streptomycin in turkeys and the emergence of streptomycin-resistant isolates from those animals (Starr & Reynolds, 1951). Since then, issues about the use of antibiotics are still questioned by the international community and professionals (Seiffert, Hilty, Perreten & Endimiani, 2013). The amount of antibiotics used at this time was considered higher than in human medicine. Addressing the rapid spread of antimicrobial resistant pathogens, Sweden was the first country to abandon the use of antibiotics as growth promoters in 1986 (WHO, 2011). In The European Union (EU) these agents were banished in the 1th January 2006 (European Comission, 2005). The effects of discontinuation of antimicrobial agents as growth promoters in European countries decreased antibiotic resistance in animals, food products, and also in humans (Anderson, Nelson, Rossiter & Angulo, 2003).

The overall amount of antibiotics used is not clearly known because the data provided from all countries is not uniform (Anderson *et al*., 2003). The amount of antimicrobial data can be

reported by weight of active ingredient or total weight of feed additives. Another important aspect is that the data reports to both companion animals and food producing animals regarding drugs licensed for multispecies used (EMA, 2009b, 2013). According to the European Medicine Agency (EMA), there is a slight downward trend for use of antimicrobials in animals in all countries. However, use of β-lactams antibiotics is increasing slightly (EMA, 2009b). The information on use and consumption of cephalosporins in the EU is not available for all members, and often the data includes β-lactam antibiotics (including ampicillin, benzylpenicillin). Furthermore, when the data provides the use of penicillins and cephalosporins separately, are not divided by generations. Currently there are specific recommendations on prudent use of cephalosporins in the EU. Nevertheless, monitorisation is needed on how the guidelines and recommendations are being implemented (Passantino, 2007).

According to data published in Portugal, the total amount of antimicrobial sold was 162,564 tons of active substance during the year of 2011 (includes all animal species). Doxycycline and amoxicillin were the most used antimicrobials. In cattle, florfenicol was the most common antimicrobial sold, and doxycycline for the swine (Direcção Geral Aimentação e Veterinária [DGAV], 2011).

2.6.2. Use of antimicrobials in companion animals

Due to the use of antimicrobials that are critical to human medicine, the risk of antimicrobial resistance emergence and inter-species clonal spread, the awareness in the last few years of the veterinary community for the potential implications for public health has increased (Martins da Costa *et al*., 2013).

Systematic surveillance of the occurrence of resistant bacteria from food animals, food and humans has been established and annually reports are published in European Food Safety Authority (EFSA). In contrast, data is rare regarding extent and characteristics of antimicrobial resistance among bacteria from companion animals (Pedersen *et al*., 2007).

The increasing number of reports on antimicrobial resistance in veterinary practice in addition to reports of multidrug pathogens led some countries to create guidelines to promote rational use of antimicrobial in veterinary practice. The Swedish Veterinary Association (SVA) developed guidelines in 2002, and countries like Denmark in 2013 followed their counterparts (SvHKS, 2013). In Sweden since 2006 a decrease in antimicrobial sales is clearly marked specially on aminopenicillins with clavulanic acid, cephalosporins and fluoroquinolones. This downward trend is explained by the application of guidelines and campaigns which led the veterinarians to change their prescription habits (SWEDRES-SVARM, 2012). According to the Danish Programme for surveillance of Antimicrobial Consumption and Resistance in Bacteria from Animals, Food and Humans (DANMAP), the use of antimicrobials in 2011

decreased in veterinary medicine due to the reduction of cephalosporins usage. The information on companion animals follows an upwards trend since 2005 caused by the use of combination penicillins (DANMAP, 2012). According to this facts Sweden is clearly a classic example of how the combination of strong policies on prescribing and implementation of guidelines can improve the rational use of antibiotics.

In the UK, a large amount of antimicrobial sales were for veterinary use (Veterinary Medicine Directorate [VMD], 2012). The total sales amount of therapeutic antimicrobials in 2008 decreased to 384 tonnes. In 2011, total sales have decreased by 101 tonnes to total 346 tonnes. Tetracyclines, β-lactams (including penicillins) and trimethoprim/sulphonamides accounted for the majority of antibiotic active ingredients sold in veterinary medicinal products from 2006 to 2011 (VMD, 2012). The incidence of *E. coli* isolates from companion animals with patterns of resistance has increased over the last years to antibiotics commonly used to treat infections caused by this agent such as amoxicillin-clavulanate, cefpodoxime, enrofloxacin, and trimethoprim sulphonamide according to a study conducted in Denmark (Pedersen *et al*., 2007).

Interestingly, pharmaceutical companies are not currently developing novel antibiotics because it is not cost effective for the drug companies. It is indeed critical to maintain the effectiveness of the antibiotic classes available by promoting a rational and prudent use (Coates & Hall, 2011). It is also important to create incentives for the research and development of new antimicrobials by industry (EMA, 2009a).

In Portugal the only extended spectrum cephalosporin approved for dogs and cats is Cefovecina (Convenia©). In dogs and cats it is indicated for skin infections caused by *S. pseudintermedius*, *Streptococci* β-haemolytic, *E. coli* and/or *Pasteurella multocida*; and for urinary tract infections caused by *E. coli* or *Proteus* spp. (only in dogs) (Associação Portuguesa da Indústria Farmacêutica [Apifarma], 2011; EMA, 2012). The use of third generation cephalosporins in human medicine in Portugal are cefetamet, cefixima, cefotaxime, ceftadizime e ceftriaxona and most can be acquired in a community pharmacy. (Infarmed, 2012). In a report published by DGAV in 2011, the most common antimicrobial used in dogs and cats was amoxicillin (DGAV, 2011). The term extra label drug use (often referred to as “off-label use”) was defined as the use in animals of a drug at an indication, dosage, frequency or route of target species (e.g. human drugs in animal species or a drug licensed for use in dogs in a cat) (Canadian Veterinary Medical Association [CVMA], 2010).

In a recent report published by The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), for the majority of countries, penicillins (36% in tonnes) were the most sold veterinary antimicrobial agent, followed by 1st and 2nd-generation cephalosporins (31%). However this data should be interpreted carefully since it only represents sales of

tablets (oral antimicrobials) and injectable antimicrobials are not included in this report (EMA, 2013).

2.6.3. Use of antimicrobial in humans

There is a clear relation between the use of antimicrobials and antibiotic resistance which is increasing in southern countries but the prevalence in northern countries remains low. Data analysis is not consensual since national databases use different methodologies to classify antibiotics and measure their consumption (Goossens, Ferech, Vander Stichele & Elseviers, 2005). In 2001, the European Commission funded the European Surveillance of Antimicrobial Consumption (ESAC) project, an international network of surveillance systems to collect comparable and reliable data on antibiotic use in Europe. The study conducted during the period 1997-2003 concluded that overall antibiotic use increased in most European countries, and penicillin was the most prescribed (Ferech *et al*., 2006). In another study by ESAC during the period 1997-2002 refers Portugal as the fourth consumer for antimicrobials in Europe with an increase of 10% in 2002 when compared with 1997. Penicillins were the most prescribed antibiotic in all countries analysed (Elseviers, Ferech, Vander Stichele & Goossens, 2007). In 2003 the study by ESAC reveals that Portugal was classified as the 4th consumer of antibiotics, and number one on fluoroquinolones usage (Ferech *et al*., 2006). Despite the downward trend in the last few years, Portugal still has a high consumption of antibiotics; and penicillin was the most prescribed agent, followed by macrolides, lincosamines, streptogramins and fluoroquinolones (Ferech *et al*., 2006).

Cephalosporins are widely used in human medicine both in hospitals and community to treat septicaemia, and meningitis (Paterson & Bonomo, 2005). The total consumption in hospitals in 15 European countries in 2002 ranged from 1.28 to 3.89 defined daily doses (DDD)/1000 inhabitants per day (Vander Stichele, Elseviers, Ferech, Blot & Goossens, 2006). In the same report an increase of third and fourth generation cephalosporin between 1997-2002 was noted in all countries (Vander Stichele *et al*., 2006).

In 2003 the total amount of antibiotics used in 34 countries European ranged from 3.89 to 31.40 DDD/1000 inhabitants per day (Ferech *et al*., 2006). Cephalosporins from third and fourth generation were limited to patients with clinical conditions difficult to control. However, in three countries the use of these cephalosporins accounted for more than 40% of the total use. These differences between countries might be explained by a misuse of the antibiotics in hospitals (Coenen et al., 2006). Defined daily dose (DDD) is a measuring unit which defines an average maintenance dose per day for a drug used for its main indication in adults (WHO, 2009).

It is crucial in all of this process to have a multidisciplinary approach together with pharmaceutical companies, agencies, professionals in the area in order to accomplish: one world, one health.

2.6.4. Prudent use guidelines

Antimicrobials are vital agents in veterinary medicine, and due to the lack of alternatives (e.g. vaccines) cannot be replaced in a near future (Ungemach, Müller-Bahrdt & Abraham, 2006). In November 2000, the German Federal Veterinarians Association (BTK) published the guidelines for prudent use of antibacterial in animals in order to reduce the use of antimicrobials. The guidelines are an important source of information in which veterinarians should comply in order to preserve human and animal health. Numerous professionals groups have developed prudent use of guidelines for treatment of animal species according to the underlying cause of infection (CVMA, 2000; Morley et al., 2005; American Veterinary Medical Association [AVMA], 2006; Ungemach *et al*., 2006). Overall the guidelines specifically outline the appropriate use of antibiotics. In the following a brief overview of the principles is given:

- Preventive strategies, such as appropriate husbandry and hygiene, routine health monitoring, and immunization, should be emphasized. Other therapeutic options should be considered prior to the use of antimicrobials;
- Prescription and extra label use of antimicrobials should always be performed by a veterinarian;
- Therapeutic use of antimicrobials is only recommended in case of bacterial infection. Substances with narrow spectrum should be chosen whenever is possible and critically important antimicrobials in human medicine should be avoided. To choose, the antimicrobial culture and antimicrobial susceptibility testing should be performed to aid selection;
- Therapeutic exposure to antimicrobials should be minimized by treating only for as long as needed for the desired bacteriological response;
- Accurate records of treatment should be maintained and analysed the outcomes to evaluate effectiveness of therapeutic regimens.

Therefore, antimicrobials can only be prescribed by a veterinarian and used by the owner according with the instructions (dosage, frequency of dosing and duration). The use of antimicrobials should be restricted for therapeutic and metaphylatic purposes. However in some cases of immunosuppressive situation or contaminated surgeries can be used as a prophylactic agent. For perioperative use, antimicrobials should have the following

characteristics: can be given intravenously to ensure a high plasma concentration; have few or no side-effects reported and should not increase the development of resistance (SvHKS, 2013).

2.6.5. Data collection methods

There are several methods for collecting data on the use of antimicrobial drugs in animals. The choice of the method is according to the specific information required. To understand and monitor the consumption of antimicrobial in animals systematic information can be collected as sales data from the pharmaceutical industries, as in the Netherlands (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands [MARAN], 2007), and the United Kingdom (VMD, 2012); from pharmacies as in Sweden and Denmark (DANMAP, 2012; SWEDRES-SVARM, 2012). Countries like Norway and Finland use their wholesaler statistics. The sales data represents an advantage in those countries; however most European countries, (Portugal included) do not have any systematic surveillance on antimicrobial usage available (Kools, Moltmann & Knacker, 2008).

Using the wholesalers or pharmacies data is useful when annually trends are analysed and allows comparing with international reality. This information can be comparable specially when using similar measuring units (e.g. kilograms) of the distributed antimicrobial.

In Sweden and Denmark data is collected from all pharmacies and all of them belong to the National Corporation of Swedish Pharmacies. All antimicrobials for companion animals are only obtainable through a veterinary prescription. Therefore annual reports on antimicrobial surveillance are available (SWEDRES-SVARM, 2012). In Denmark the antimicrobial usage is controlled by the Danish VetStat® system which collects all the information from the pharmacies. Information such as animal species and dosage can be obtained by prescription data (DANMAP, 2012).

As stated above, the information provided by wholesalers or pharmacies gives important information concerning the usage of antimicrobial in different countries. The implementation of guidelines is critical, however is not mandatory, and the veterinarians should adopt these to help control the spread of resistant bacteria. To understand if the veterinarians are following the guidelines implemented it is necessary to document if the actual prescriptions are according to the recommendations (Regula, Torriani, Gassner, Stucki & Müntener, 2009).

Antimicrobial use in animals can be monitored via national data on import or sales of antimicrobials, via pharmacies. National sales data are used most often, because they are relatively easy to obtain. However the indication for the treatment is not included in these data and its considered critical to understand if the antimicrobials are used prudently (Regula *et al*., 2009). In a study conducted in Switzerland, Regula (2009) reviewed the use of

antimicrobials since these agents are sold directly from distributors to veterinary practices. In companion animals, cephalosporins and β-lactams represented the majority of all antimicrobials used. Many of the criteria stated in recommendations for prudent usage of antimicrobials could not be evaluated with the data available in this study and therefore only analysed the compliance of veterinarians to the prudent use of antimicrobials (Regula *et al*., 2009).

Antimicrobial agents licensed for human use are also used frequently in companion animals (in application of the 'Cascade') (Arts. 10 & 11 of Directive 2001/82/EC of the European Parliament and of the Council). Such data is included in the sales data for human antimicrobial agents (EMA, 2013). The cascade is a legal flexibility providing a rational balance between the legislative requirement for veterinarians to prescribe and use authorized veterinary medicines where they are available, and the need for professional freedom to prescribe other medicines where they are not. It is intended to increase the range of medicines available for veterinary use particularly when welfare of the animal patient could be compromised otherwise (British Small Animal Veterinary Association [BSAVA], 2013). According to the cascade, the veterinarian may treat the animal(s) in order to avoid unacceptable suffering, in accordance with the following: a veterinary medicine authorized from another animal species or for a different condition within the same species. If that medicine does not exist, use either a medicine approved for human medicine, or a veterinary medicine authorized from another Member State of the EU. In case that there is no such medicine, a medicine prepared extemporaneously can be used (Decreto-lei n. º 148/2008, alterado pelo Decreto-lei 314/2009, artigo 78 [Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, 2008]. In this sense, the prescribing cascade promotes the responsible antimicrobial use, allowing the veterinarian to use clinical judgement to prescribe a drug if no veterinary authorised medicine exists (BSAVA, 2013).

In Canada, Weese (2006) performed a retrospective study on prescription for dogs and cats at a veterinarian teaching hospital in Ontario, reviewing 10 years of prescribing data (1995-2004). Overall, 21.152 antimicrobial prescriptions were written for dogs and cats. Extent of antimicrobial prescriptions was considered lower than in previous studies performed in Canada. The use of first-generation cephalosporins, fluoroquinolones, penicillins and trimethoprim-sulphonamides decreased over time; while the use of metronidazole increased Factors such as increased awareness regarding consequences of overuse antimicrobials as a consequence of the guidelines introduced may have had an important impact. In this study the underlying disease for which antimicrobials were prescribed was not investigated, compliance with prudent use guidelines could not be evaluated but requires equal attention (Weese, 2006).

2.7. Mechanisms of antimicrobial resistance: Vertical and Horizontal gene transfer

Several mechanisms are responsible for antibacterial resistance. Bacteria can be innately resistant to a specific class of antimicrobial, and all descendants from these bacteria are also resistant, named vertical transfer of resistance (Woodford & Ellington, 2007; Bennett, 2008). When mutation occurs two scenarios may happen. In one hand the bacteria might only reduce the susceptibility to a specific antimicrobial agent to allow the survival until new resistant genes are acquired. In the other hand, in some rare cases a single mutation can be enough to confer high level resistance (e.g. high-level rifampicin resistance in *S. aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni* (Tenover, 2006). Antibiotic resistance genes are a major problem and several studies have focused on this issue trying to understand what is happening in the core of the bacteria for these to become increasingly resistant. It became clear that the mutation could not be the only cause. Further studies were required and the attention is now focused on the role of transferable genetic material from other resistant organisms (e.g. acquired resistance). This is termed horizontal gene transfer and may occur between strains of the same species or between different bacterial species or genera (Tenover, 2006). Horizontal transfer can be defined as a process in which DNA bacterial is transferred from one cell to other and stay stably incorporated in the other cell genome, without cell division (

Figure 6) (Carattoli, 2003).

The DNA can be transferred from one cell to other by three methods: transformation, transduction and conjugation, and in these processes new gene (s) from other bacteria are acquired. The conjugation process includes plasmids and conjugative transposons which are able to transfer genetic material from one cell to other involving replication. To accomplish this, the donor bacteria extend an elongated structure named *pilus* that connects with the recipient bacteria. It is necessary that the donor cell and recipient are in direct contact for the success of this method. The other elements involved in the conjugation are resistance transposons, gene cassettes and inserted sequences (IS) which can transfer genetic material within the same cell (e.g. from chromosome to plasmid) by recombination process (Bennett, 2008). Transposons are small, mobile DNA elements which include insertion sequences and have the ability to move intra and inter DNA molecules inside the cell. The resistance genes located in plasmids are often placed in transposons which contain the transposase function to facilitate the transposon to recombine into the chromosome or plasmid. The importance of transposons in the increase of antibiotic resistance relies on the fact that most of them encode resistance to tetracycline or minocycline alone (Rice, 1998; Carattoli, 2003; Seiffert *et al*., 2013). Some transposons are conjugative while others require mobile elements such as plasmids (Seiffert *et al*., 2013).

Plasmids, especially conjugative plasmids are genetic mobile elements that move bacterial genes from one cell to other. In general, they are autonomous from the host, replication process independent, and do not need any set of core genes to start replication, but may carry genes that from an evolutionary perspective can be useful for their survival. Plasmids are circular, double stranded DNA. A resistant plasmid is defined as a plasmid that carries one or more antibiotic resistant genes (Bennett, 2008). According to Salvador Luria, plasmids are "among the most fundamental advances in the whole history of bacteriological science" (Luria, 1947).

Integrons can be localised whether in the chromosome or in plasmids. These elements can incorporate single genes and they contain an integrase and an attI binding site for the integration on cassettes. The cassettes have an attC repeated sequence in the flanks and enables to go to be integrated at the attI site, excised and undergo horizontal gene transfer (Seiffert *et al*., 2013). Integrons are an important vector since they can carry up to eight resistant gene cassettes (Naas, Mikami, Imai, Poirel & Nordmann, 2001). Other aspect is that any gene can be assimilated in an integron gene cassette which represents an advantage for the bacteria (Rowe-Magnus & Mazel, 2002). Four classes of integrons have been described. Class 1 and class 2 are the most prevalent among bacteria such as *E. coli* and *Salmonella.* These integrons are disseminated in companion animals and food-producing animals. As these integrons are similar to those found in human commensal and pathogens it is possible to establish the hypothesis of lateral transfer between humans and animals (Stokes & Gillings, 2011).

Acquisition resistant genes in Enterobacteriaceae is mainly due to plasmids, but some studies have pointed out the significance of the ICEs (integrative and conjugative elements) as a factor contributing to the dissemination of resistance genes (Mata *et al*., 2011). ICEs are mobile genetic elements found in the chromosome of both gram-positive and gram-negative bacteria and can move from the chromosome of one cell to other by meanings of excision, and transferred by conjugation (Wozniak & Waldor, 2010).

During transduction, the DNA transfer through bacteria from the same species is mediated by bacteriophages (bacterial viruses), but it is limited to large and double stranded phages (50-100kb). Within the bacteria, the bacteriophages can remain inactive as prophages or become virulent and start replication which results in maturation of the virus particles and can pack his own DNA or acquire it from the host (Frost, Leplae, Summers & Toussaint, 2005).

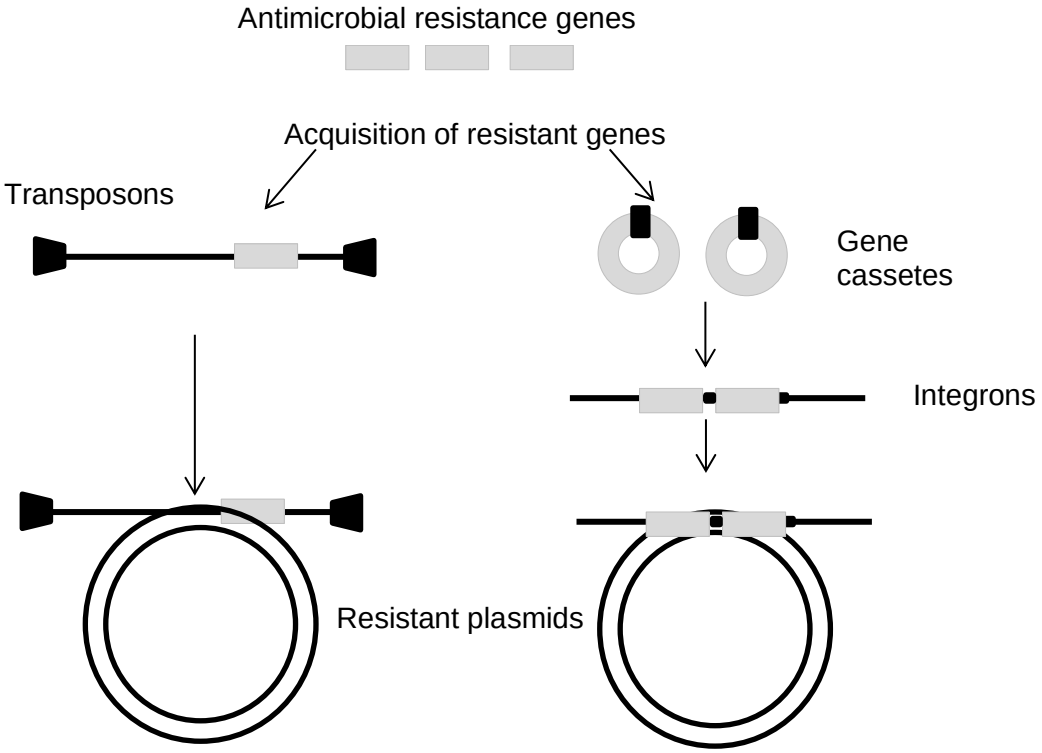
In transformation, the bacteria acquires and incorporates DNA segments which are in the environment and were released by bacteria after cell lysis (Tenover, 2006). This process was the first mechanism of prokaryotic HGT to be discovered (Frost *et al*., 2005).

It is not easy to measure the importance of HGT because there is a weak knowledge of the contributing factors and there are some difficulties to reproduce them. Another characteristic

is that only a limit number of species are able to be transformable. This process is mainly used to monitor the transfer of antibiotic resistant genes (Rizzi *et al*., 2008).

As a consequence of these resistance mechanisms, the bacteria can adapt easily to the introduction of new bacterial genes and become resistant (Carattoli, 2003).

Figure 6 - Scheme representing the acquisition of plasmids resistance genes (adapted from Carattoli, 2003).



2.8. Resistance to β-lactams

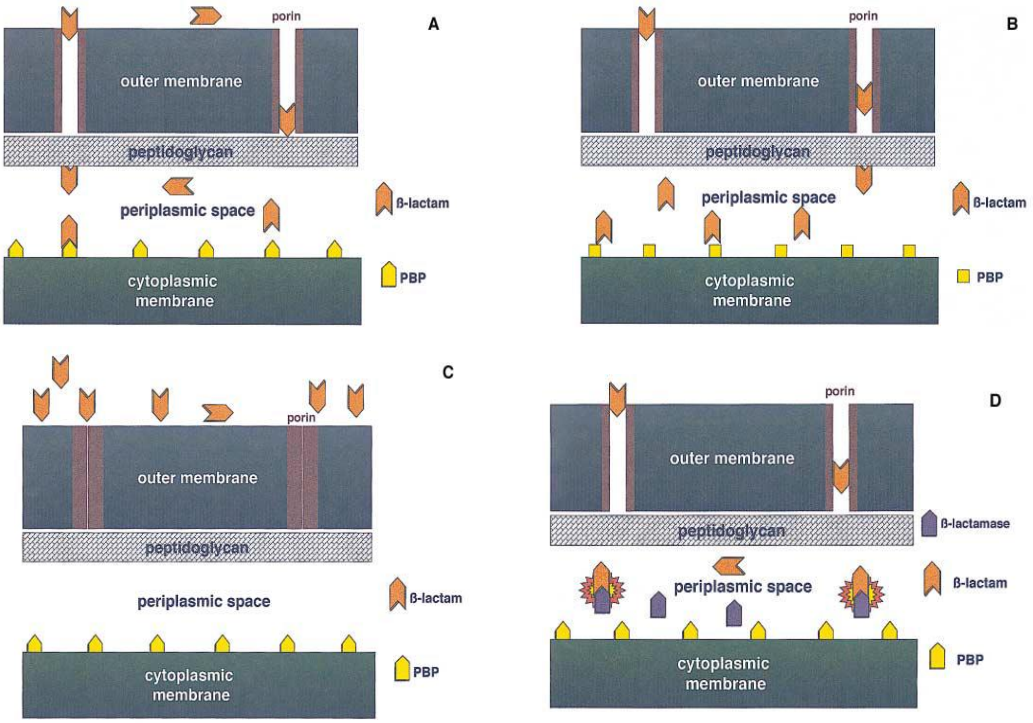
Over the past 60 years, due to the use of β-lactam antibiotics, the production of β-lactamases enzymes among gram-negative organisms has increased, especially in Enterobacteriaceae (Livermore & Woodford, 2006). The introduction of third generation cephalosporins started with cefotaxime 30 years ago and it improved the antimicrobial chemotherapy in humans and veterinary medicine. Undoubtedly, as a consequence of the selective pressure exerted by these new cephalosporins, resistance in enterobacterial species emerged a few years later (Pfeifer, Cullik & Witte, 2010). Bacteria became resistant through one of the three primary mechanisms of resistance. The first mechanism and also the most common is the production of β-lactamase enzymes that hydrolyse or modify the antibiotic before reaching his target site (Figure 7). The β- lactamase breaks a bond in the β-lactam ring to disable the molecule, so the bacteria remain resistance to any β-lactam antibiotic. The second mechanism is alteration of the antibiotic target site, and these mutant PBPs have low affinity towards β-

lactam antibiotics; the last mechanism is the alteration of permeability or forced efflux that inhibits the antibiotic to reach the target (Poole, 2004; Wilke *et al*., 2005).

With the increase of β-lactamase producing Enterobacteriaceae in the past 20 years new β-lactam antibiotics have been developed specifically to fight against the hydrolytic action of β-lactamases (Medeiros, 1997). The first detection of ESBLs from an animal was reported in 1988 in Japan isolated in an *E. coli* from a laboratory dog (Matsumoto, Ikeda, Kamimura, Yokota & Mine, 1988). Since then, numerous reports have been made of ESBL producing bacteria across Europe, and it is a major problem worldwide (Carattoli *et al*., 2008).

To date, more than 1000 β-lactamases have been reported as mentioned in http://www.lahey.org/studies/webt.htm.

Figure 7 - Resistance to β-lactam antibiotics: (A). In the gram-negative cell β-lactam antibiotics action (B); modification of the targets of the drugs, (C); the PBPs alterations in porin proteins, (D); production of β-lactamases (reproduced with permission from Pitout, Sanders & Sanders, 1997).



The main mechanism of acquired resistance to extended-spectrum cephalosporins in Enterobacteriaceae is the production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) and/or plasmid-mediated AmpC β-lactamases, and *E. coli* is one of the major producers (Tamang *et al*., 2012). The ESBLs have been isolated from a wide variety of Enterobacteriaceae (Bradford, 2001).

The ESBLs are defined as a β-lactamases capable of hydrolysing penicillins broad, extended spectrum cephalosporins, monobactams and are inhibited by clavulanic acid (functional

group 2 be). The AmpC type β-lactamases which are also commonly isolated from extended-spectrum cephalosporin-resistant gram-negative bacteria are different from ESBL because they are not inhibited by clavulanic acid or other β-lactamase inhibitor. AmpC β-lactamases are typically encoded in the chromosome or in plasmids from many gram-negative bacteria (Bush, Jacoby & Medeiros, 1995). The β-lactamases are located in the periplasmatic space in gram-negative bacteria, whereas in gram-positive are mostly extracellular (Bush, 2010).
The rapid dissemination of isolates producing ESBLs is considered a major concern due to the fact that these plasmids frequently carry genes encoding resistance to other drug classes as fluoroquinolones, aminoglycosides, sulfa-derivatives and trimethoprim (Pitout & Laupland, 2008; Hawkey & Jones, 2009).

2.8.1. Genetics of β-lactamase: inducible or constitutive

The enzymes encoded in the chromosome can be inducible or have constitutive expression. Inducible means that the enzyme is expressed in the presence of a substance (an inducer), and constitutive expression means that a gene is transcripted continuously (Mayer, 2010). In Enterobacteriaceae, AmpC enzymes encoded in the chromosome are usually inducible (Jacoby, 2009; Bush, 2010;). In Table 3 are listed the AmpC β-lactamases producing organisms inducible by β-lactam antibiotics such as cefoxitin but poorly induced by the third and fourth generations cephalosporins (Jones, 1998; Hanson, 2003). The induction of AmpC gene includes the gene products AmpR, AmpD, and AmpG (Hanson & Sanders, 1999).
The increase of β-lactam resistance in Enterobacteriaceae is a consequence of AmpC expression gene which encodes a cephalosporinase very common in *E. coli* and *E. cloacae*, but with different mechanism patterns (Honoré, Nicolas & Cole, 1986). In *E. coli* the expression of AmpC gene is regulated by a weak promoter and a transcriptional attenuator, producing a small amount of enzyme and bacteria are susceptible to ampicillin (Olsson, Bergström, Lindberg & Normark, 1983). But the wild strain can undertake some alterations and hyper-produces AmpC gene by mutation in a promotor-operator region determining the rate of transcription from the structural gene AmpC (Bergström & Normark, 1979). Molecular studies evidenced that some strains might have more than one copy of the AmpC gene (Nelson & Elisha, 1999). In this case these strains are resistant to practically all β-lactams, with the exception of carbapenems (Garau, 1994).

Table 3 - Enterobacteriaceae producing inducible AmpC β-lactamases (adapted from (Jones, 1998)

Genus	Species
Citrobacter	*Freundii*
Enterobacter	*aerogenes, cloacae*
Morganella	*Morganii*
Hafnia	*Alvei*
Serratia	*Marsecens*

2.8.2. Transferable resistance

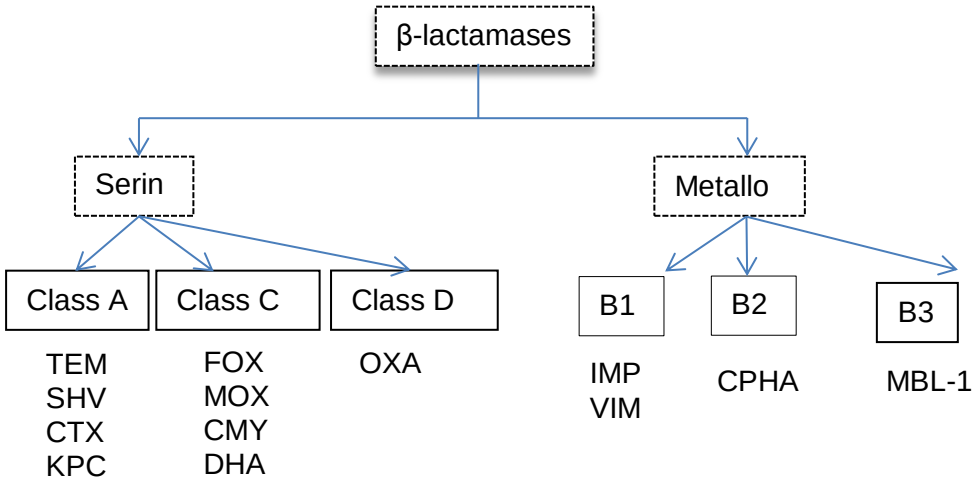
The dissemination of β-lactamases is due to their location within plasmids and transposons which facilitate the transference of resistant genes among bacteria (Bradford, 2001). In this sense bacteria gains advantage over antibiotic use, by introducing AmpC enzymes from the chromosome into plasmids (Jacoby, 1994). There are several reports in humans, cattle and dogs described (Winokur *et al*., 2000; Sanchez *et al*., 2002).

2.9. Most important β-lactamases

In Enterobacteriaceae, the major mechanism of β-lactam resistance is the production of β-lactamases (Majiduddin, Materon & Palzkill, 2002). This resistance mechanism is often associated with mobile genetic elements such as insertion elements, transposons and integrons, and is highly selected by the selective pressure caused by the use of β-lactam antibiotics (Massova & Mobashery, 1998).

Since early 1970s different classifications based on phenotype, gene or amino acid protein sequences and function have been described (Ambler, 1980; Bush *et al*., 1995). Classification of β-lactamases can be done according to the functional properties (Bush-Jacoby-Medeiros scheme) defined by the substrate and inhibitor profile (Table 4). There are four main groups and multiple subgroups in this system. This classification scheme is of much more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant (Paterson & Bonomo, 2005). Group 1 includes cephalosporinases that are not well inhibited by clavulanic acid; group 2 refers to penicillinases, cephalosporinases, and broad-spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors; The last group, group 3 includes the metallo β-lactamases (MBL) that hydrolyse penicillins, cephalosporins, and carbapenems and that are poorly inhibited by almost all β-lactams (Bush *et al*., 1995). In contrast, the Ambler classification scheme divides the β-lactamases into 4 classes (A, B, C and D) upon their amino acid sequence and not phenotypic characteristics (Figure 8). Class A, C, and D all have a serine at their active site, while class B are metallo enzymes that require at least one active site zinc ion to facilitate β-lactam hydrolysis (Hall & Barlow, 2005; Paterson & Bonomo, 2005; Bush & Jacoby, 2010).

Figure 8 - Current Ambler classification scheme (adapted from Hall & Barlow, 2005; Papp-Wallace, Endimiani, Taracila, & Bonomo, 2011; Poole, 2004)



2.9.1. TEM ESBLs type

According to Amber classification, the class A β-lactamases is the most common group and consists of TEM, SHV and CTX-M β-lactamases enzymes (Bush *et al*., 1995). Most ESBLs are derivatives of TEM or SHV enzymes and there are over 200 TEM types β-lactamases described, as reported in http://www.lahey.org/studies/webt.htm.

Both TEM and SHV-type ESBLs are often found in *E. coli* and *K. pneumonia*, however, they have also been found in *Proteus* spp., *Providencia* spp., and other genera of Enterobacteriaceae (Bradford, 2001).

The first plasmid-mediated β-lactamase in gram-negative bacteria, TEM-1, was described in the early 1960s from a patient in Athens named Temoneira (hence the designation TEM) and it is actually the most common β-lactamase encountered in gram-negative bacteria (Bradford, 2001; Kaur & Aggarwal, 2013). TEM-1 can be found in many different species of the family Enterobacteriaceae, such as, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* due to the fact that is plasmid and transposon mediated (Bradford, 2001). Enterobacteriaceae express frequently plasmid encoded broad-spectrum-β-lactamases such as TEM-1, TEM-2 which confers resistance to amino-penicillins and first generation penicillins, but not to third or fourth generation cephalosporins and are inhibited by clavulanic acid. The TEM-3 type ESBL derivate from TEM-1 and TEM-2 (Chaibi, Sirot, Paul & Labia, 1999; Bradford, 2001; Paterson & Bonomo, 2005). TEM-2 is referred as the first enzyme derivate from TEM-1 and it only has one single amino acid substitution. Both TEM-2 and TEM-3 have the same hydrolytic profile of TEM-1 (Barthélémy, Peduzzi & Labia 1985). The first TEM-type β-lactamase that displayed the ESBL phenotype was TEM-3 reported primarily in 1989 (Bradford, 2001).

Although the inhibitor-resistant β-lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM or SHV-type enzymes. These

enzymes were named first IRT for inhibitor-resistant TEM β-lactamase; however, all have subsequently been renamed with numerical TEM designations (Bradford, 2001).

2.9.2. SHV – Type ESBLs

The first report was discovered from a *Klebsiella ozaenae* isolate in Germany as a chromosomally encoded β-lactamase. The name SHV designates a contraction of sulphydryl variable. Subsequent work demonstrated that SHV-1 is identical to the PIT-2 enzyme which was described in 1972, encoded by a transposon and found on plasmids of different incompatibility types (Heritage, Gascoyne-binzi & Hawkey, 1999). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Paterson & Bonomo, 2005). The first report of SHV enzyme had a narrow spectrum, with activity against penicillin, capable of hydrolyse cefotaxime and ceftazidime. Some mutations have occurred in the active site of the enzyme which led to other SHV enzymes. These derivate either have an extended-spectrum activity towards third generation cephalosporin or are resistant to β-lactamase inhibitors (Knothe, Shah, Krcmery, Antal & Mitsuhashi, 1983; Heritage *et al*., 1999) . The use of third generation cephalosporins can be responsible for the increase and dissemination of organisms harbouring SHV-2 (Paterson & Bonomo, 2005).

SHV- type β-lactamases which are most commonly isolated from *K. pneumonia* are often encoded on large transferable plasmids, reported worldwide and more than 120 types have been described (http://www.lahey.org/Studies) (Du Bois, Marriott & Ajnyes, 1995; Bradford, 2001).

2.9.3. CTX - Type ESBLs

In the past decade, CTX enzymes have become the most prevalent extended-spectrum β-lactamases worldwide in healthcare and in community settings. They also have been described in farm animals, pets, products from food chain and sewage (Cantón & Coque, 2006). This family of ESBLs enzymes were first described in the late 1980's. The name CTX reflects the potent hydrolytic activity of these β-lactamases to cefotaxime. The M in the CTX-M refers to the city of origin, Munich (Paterson & Bonomo, 2005). These enzymes are defined by their capacity to cause resistance to cefotaxime and susceptibility to ceftazidime (Pitout, 2008)**.** A notable exception to this is CTX-M-15, the most common CTX-M variant which can also cause resistance to ceftazidime in addition to cefotaxime. The CTX-M family is a heterogeneous group which had rapidly spread over the last decade and have become the most prevalent ESBL (Cantón & Coque, 2006). There are currently more than 100 different CTX-M enzymes that can be divided into six different subgroups based on their amino acid sequences: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and CTX-M-45 (www.lahey.org/studies) (Rossolini, D'Andrea & Mugnaioli, 2008)**.**

2.9.4. Metallo β-lactamases

The class B or metallo β-lactamases inactivate the β-lactam antibiotic cleaving the amide bond; hydrolyse all β-lactams antibiotics except monobactams. In addition, these enzymes have an efficient activity against carbapenems and are not susceptible to β-lactam inhibitors (Bebrone, 2007). The first enzyme identified was found in an opportunist environment bacteria of *Bacillus cereus* in 1966, and it was considered clinically unimportant, because the metallo β-lactamases genes were intrinsic and chromosome born (Nordmann *et al*., 2012). The metallo β-lactamase resistant genes are located in the chromosome mainly but can be also detected in plasmids and integrons (Bebrone, 2007). The plasmid confers the possibility of horizontal transfer among pathogenic and/or opportunistic bacteria leading to a serious clinical problem (González, Martín, Costello, Tierney & Vila, 2007). The increasing reports of MBL led to subsequent studies to evaluate and group them into three subclasses B1, B2 and B3 on the basis of their sequence alignments. The group B1 and B3 structurally can bind up to two Zinc ions and are capable to hydrolyse most β-lactam antibiotics including penicillins, cephalosporins and carbapenems while group B2 only has one zinc ion and is very active against carbapenems (González *et al*., 2007).

The most important carbapenemases are categorized as three types of enzymes: (i) the KPC (*K. pneumoniae* carbapenemases) type enzymes (ii) the VIM (Verona integron encoded metallo β-lactamase), IMP (for “active on imipenem”), and NDM (New Delhi metallo-β-lactamase) metallo-β-lactamases; and (iii) the OXA-48 type enzymes. The most common types found in Enterobacteriaceae are IMP and VIM groups (Queenan & Bush, 2007). The IMP metallo β- lactamase was first reported in 1991 in Japan from a *Serratia Marsecens.* These have been progressively disseminating since then to other countries and into various Enterobacteriaceae genus *(Serratia marcescens, Klebsiella pneumoniae, Citrobacter freundii), Pseudomonas aeruginosa,* and other non-fastidious gram-negative non-fermenters (Nordmann *et al*., 2012).

2.9.5. AmpC β-lactamases

AmpC β-lactamases were the first enzyme described to destroy penicillin and was reported in *E. coli*. The structural gene for the production of the β-lactamase was named *bla*AmpC and the sequence of the gene from *E. coli* was described in 1981. In Amber classification the AmpC enzymes are classified as class C whereas according to Bush are incorporated in group 1 based on the functional classification scheme (Bush & Jacoby, 2010). The molecular masses of typical AmpC enzymes vary from 34 to 40 kDa, the isoelectric points of > 8.0 and are generally located in the bacterial periplasm. AmpC enzymes are active against penicillin, their activity is higher towards cephalosporins, with the exception of 4th generation

cephalosporins (cefepime and cefpirome); hydrolyse cephamycins, like cefoxitin and cefotetan, and monobactams. In opposition to ESBLs, AmpC enzymes are poorly inhibited by clavulanic acid, sulbactam, and tazobactam, and not at all by Ethylenediaminetetraacetic acid (EDTA) (Jacoby, 2009).

Since the 1970s, the AmpC β-lactamases have been a target of study (Hanson, 2003). The AmpC β-lactamases were encoded mainly in the chromosome in various gram-negative bacteria e.g. as *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *P. aeruginosa*, and *Hafnei alvei* (Medeiros, 1997). In many Enterobacteriaceae, AmpC expression is low but inducible in response to β-lactam usage (Jacoby, 2009). The induction of AmpC β-lactamases is controlled by the activity of three proteins: AmpG, AmpD, and AmpR. Usually in a wild type strain, AmpC production is expressed at low levels, but when mutation occurs in the AmpD and AmpR results in AmpC overexpression or constitutive hyper production and increases the β-lactam MICs in organisms with an inducible AmpC (Schmidtke & Hanson, 2006). The mechanism most associated with constitutive overproduction of AmpC is due to amino acides substitution in the AmpD (Hanson & Sanders, 1999). The hyper production of AmpC β-lactamases results in resistance to most β-lactam antibiotics with the exception of cefepime, cefpirome, and the carbapenems (Thomson & Moland, 2000). The amount of enzyme that is inherited depends on the species on their regulation mechanism. In *E. coli* the production of this enzyme is in the chromosome, usually repressed and at low levels, meaning that AmpC is non-inducible (Jacoby, 2009). However if mutations in the promoter region occurs there is a hyper production of the enzyme with levels of resistance to ampicillin and cephalosporins (Jacoby, 2009). Another important pathogen *Salmonella* spp. does not carry this type of enzyme inherently, but in recent years genes encoding CMY enzymes have been described on plasmids (Arlet *et al*., 2006). Also *Proteus* spp. and *K. pneumoniae* normally do not harbour chromosomal bla_{AmpC} genes (Drawz & Bonomo, 2010). Class C AmpC β-lactamases include CMY-2, ACT-1, and DHA-1, which are usually encoded by *bla* genes located on the bacterial chromosome (Drawz & Bonomo, 2010).

In Enterobacteriaceae the genes encoding AmpC were mobilized from the chromosome to plasmids and now have spread horizontally between different species of Enterobacteriaceae and are becoming more prevalent (Alvarez, Tran, Chow & Jacoby, 2004). The mobilization of AmpC genes into plasmid is due to genetic elements as the insertion sequence IS*Ecp1* ,which is associated with many CMY alleles including CMY-2 (Haldorsen *et al*., 2008).The plasmid location of this enzyme is usual responsible to encode resistant to other antimicrobials class, as fluoroquinolones, aminoglycosides, sulphonamides and trimethoprim, as well as genes for other β-lactamases (Alvarez *et al*., 2004). As a consequence, treatment options are limited and increases the burden of antimicrobial resistance (Pitout & Laupland, 2008).

Plasmid-mediated AmpC β-lactamases were reported first in 1988 and derived from older, broad-spectrum β-lactamases (e.g. TEM-1, TEM-2,SHV-1), which have an extended substrate profile, similar to the chromosomally determined AmpC β-lactamases that permits hydrolysis of all cephalosporins, penicillins, and aztreonam (Thomson, 2001). Susceptibility to cefepime, cefpirome, and carbapenems is little, if at all, affected (Jacoby, 2009). The exception is that ACC-1 does not confers resistance to cephamycins and is actually cefoxitin inhibited (Bauernfeind, Schneider, Jungwirth, Sahly & Ullmann, 1999).

Plasmid-mediated AmpC β-lactamases have been reported around the world in both animal, human and nonnosocomial isolates, having been most easily detected in those Enterobacteria not expected to produce AmpC β-lactamases (Jacoby, 2009). These enzymes exhibit high similarity with chromosomally determined AmpC β-lactamases, and thus probably represents the progenitors for the plasmid encoded enzymes (Jacoby, 2009). Plasmid-mediated AmpC β-lactamase (pAmpC) includes several families which exhibit little differences between them, and are grouped into nine groups, including 90 CMY alleles, 13 variants of ACT and 10 of FOX, 8 variants of DHA and MOX, 5 of MIR and ACC, and CFE-1 and LAT-1 (http://www.lahey.org/Studies/).

One of the concerns about plasmid-mediated resistance to β-lactams is that often encode additional resistance to other antimicrobial class, as aminoglycosides, chloramphenicol, fluoroquinolones, sulphonamides, tetracyclines, and trimethoprim (Alvarez *et al*., 2004). The most common plasmid-mediated AmpC β-lactamase worldwide is CMY-2, and to date more than 100 alleles have been reported (http://www.lahey.org/Studies/). In annex 2, the main characteristics of bla_{CMY-2} gene are illustrated. CMY-2 gene is not inducible while other genes for ACT-1, DHA-1, DHA-2, and CMY-13 are linked to AmpR and are inducible (Jacoby, 2009). CMY gene has two different origins and six varieties (CMY-1, 8, 9, 10, 11, and 19), are related to chromosomally determined AmpC enzymes in *Aeromonas* spp., while the others (including CMY-2) are related to AmpC β-lactamases of *Citrobacter freundii* (Barlow & Hall, 2002).

The plasmid encoded enzyme can be a hard task to identify since phenotypic identification is difficult and it can be misidentified as extended-spectrum β-lactamases (Hanson, 2003). There are no guidelines to detect plasmid encoded AmpC mediated resistance in gram-negative organisms. When an organism shows resistance to cefoxitin can indicate the possibility of AmpC producer but can also indicate reduce outer membrane permeability (Thomson, 2001; Jacoby, 2009). To detect all ESBLs and AmpC-type β-lactamases according to EFSA, testing cefotaxime and the use of epidemiological cut-off values should be sufficient. It is important to distinguish cefoxitin AmpC producers from cefoxitin resistant non-AmpC producers, because therapy options are different. In the first case carbapenems would be the treatment of choice and extended-spectrum cephalosporins would be

recommended for the latter (Coudron, Hanson & Climo, 2003). A multiplex PCR for the detection of plasmid encoded ampC genes was developed by Pérez-Pérez & Hanson (2002), a rapid and useful test to distinguish between AmpC producers and non-producers (Pérez-Pérez & Hanson, 2002).

The concern among researchers is based on the fact that these enzymes can be plasmid-mediated in gram-negative, and have been found not only in humans but also in livestock and companion animals. These findings address the question about surveillance in order to control the spread of resistant genes in gram-negative bacteria (Hanson, 2003).

Table 4 - β-lactamases classification scheme (adapted from Bush & Jacoby, 2010).

Classification by Jacoby- Medeiros				Inhibited by	
Group	Molecular class	Substract	Characteristics	CA/TZB [a]	EDTA[b]
1	C	Cephalosporins	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	NO	NO
1e	C	Cephalosporins	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	NO	NO
2a	A	Penicillins	Greater hydrolysis of bezylpenicillin than cephalosporins	YES	NO
2b	A	Penicillins, early cephalosporins	Similar hydrolysis of benzylpenicillin and cephalosporins	YES	NO
2be	A	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	YES	NO
2br	A	Penicillins	Resistance to clauvulanic acid, sulbactam, and tazobactam	NO	NO
2bre	A	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	NO	NO
2c	A	Carbenicillin	Increased hydrolysis of carbenicillin	YES	NO
2ce	D	Carbenicillin, cefepime	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	YES	NO
2d	D	Cloaxillin	Increased hydrolysis of cloaxcillin or oxacillin	Variable	NO
2de	D	Extended-spectrum cephalosporins	Hydrolyses cloaxcillin or oxacillin and oxyimino-β-lactams	Variable	NO
2df	D	Carbapenems	Hydrolyses cloaxicillin or oxacillin and carbapenems	Variable	NO
2e	A	Extended-spectrum cephalosporins	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	YES	NO
2f	A	Carbapenems	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	Variable	NO
3a	B (B1)	Carbapenems	Broad-spectrum hydrolysis including carbapenems but not monobactams	NO	YES
3b	B (B2)	Carbapenems	Preferential hydrolysis of carbapenems	NO	YES

[a] CA - Clavulanic acid; TZB – Tazobactam.

[b] EDTA - Ethylenediaminetetraacetic acid.

Part 2

Study 1

3. Use of antimicrobials in companion animals

3.1. Aim of this study

In several countries, the monitoring programs are focused on food-producing animals. In general most countries have some data about the antimicrobial use in animals but it's not very accurate in companion animals. According to the directive EU 2004/28/EC it is mandatory that all member countries compile detailed antimicrobial data. Some countries have this task easier since the prescription antibiotics are surrendered by pharmacies and are included in their annual reports (DANMAP, 2012; SWEDRES-SVARM, 2012). In Portugal for instance, the pattern of prescription is unknown and there is no national monitoring program to follow the antimicrobial usage in companion animals. The lack of data in this area led to the creation of this survey in order to gather information, quantify and characterise the patterns of antimicrobial usage in companion animals. The rational use of antimicrobials in small animals should become second nature to veterinarians. Since most veterinary practices do not perform bacterial culture and susceptibility testing before antibiotic prescription, with this project another aim is to raise the awareness of veterinarians for the importance of performing bacterial culture and testing antimicrobial susceptibility, which will support a better surveillance on antimicrobial resistance and will guide selection of antimicrobial therapy.

The specific aims of the study were:

(i) To analyse the patterns of antimicrobial prescription from veterinarians in small animal practice, in Portugal;

(ii) To determine the use and/or possible misuse of antimicrobials in companion animals by comparing the results with guidelines and previous studies.

3.2. Material and methods

3.2.1. Validation of the survey

Initially a pilot study was performed. The questionnaire was tested on 30 people (including veterinarians and pet owners) randomly selected, in order to validate and identify potential problems. The pilot study was successful and no changes were made to questionnaire.

The survey was sent by the OMV to all veterinarians with an active subscription, to a total of 4800 veterinarians.

3.2.2. Data collection

The survey was available in an online platform, named SurveyMonkey® accessible at www.surveymonkey.com. The veterinarians were asked to answer the survey, were informed of the purpose of this study and were kindly requested to leave their email address in case they would like to know the results of this study. This survey was online since the 19th November 2012 until 1th March 2013.

The questionnaire was composed of three sections (see annex 3) the first section refers to socio-demographic data from the respondents (age, gender, year of practice, specialization). The second section included the antimicrobial prescribing practices (sources of information for antimicrobials, importance of clinical signs, microbiological culture and weight when prescribing antimicrobials). In the last section of the questionnaire, the clinicians were requested to choose according to the underlying disease which antimicrobial would be prescribed to assess appropriateness of antimicrobial selection.

3.2.3. Statistical analysis

The data was collected in a Microsoft® Excel 2007.

3.3. Results

3.3.1. Response rate

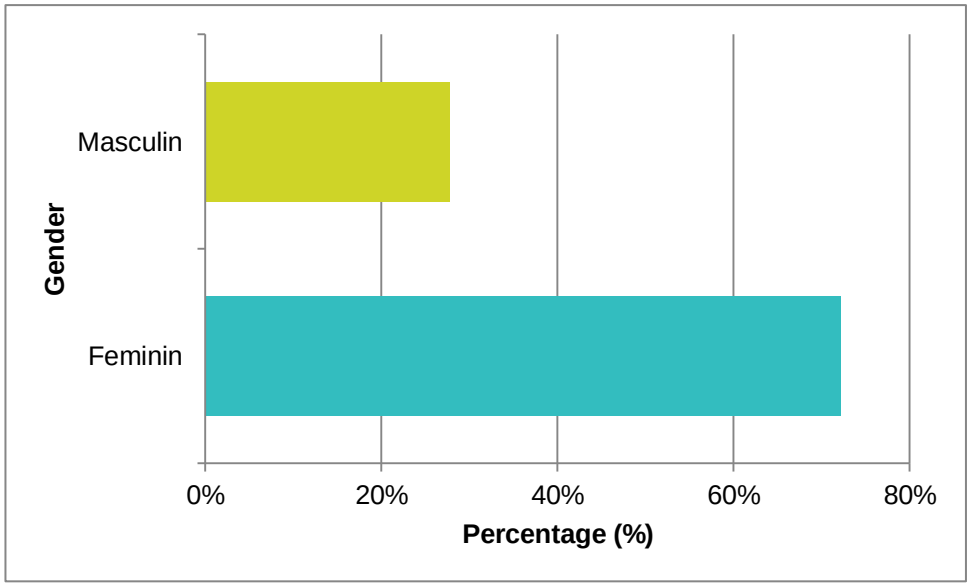
A total of 183 surveys (n=183) were started. Eighty one of the answered questionnaires were not completed, leaving 102 completed questionnaires and a useable response rate of 3%.

3.3.2. Questionnaire based analysis

The majority of veterinarians who responded to the questionnaires were females (*n*= 130; 72%), while man accounted for 28% (*n*= 30), as illustrated in Figure 9. Most of the veterinarians worked in a Veterinary Clinic (*n*= 91; 51%), 22% (*n*= 39) worked in a Veterinary Practice and 17% (*n*= 31) worked in a Veterinary Hospital (data not shown).

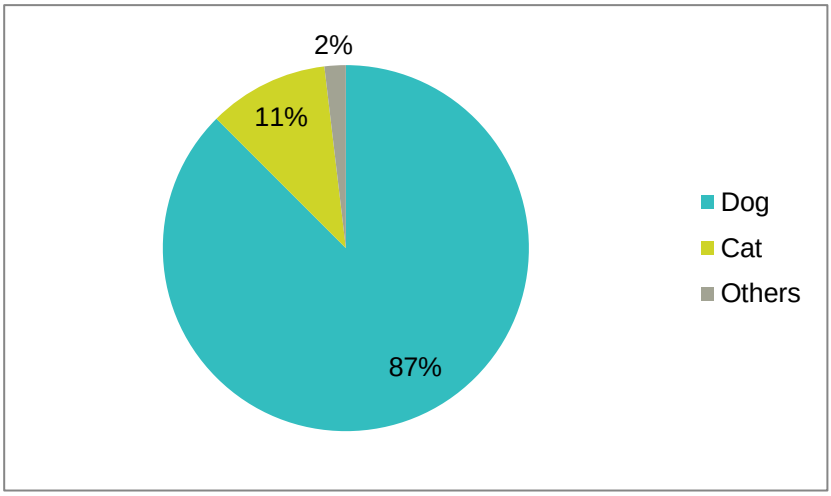
Regarding their qualifications, most of the veterinarians (*n*= 114; 64%) did not hold any post graduate qualifications while 37% (*n*= 66) hold post-graduate qualifications but no details were provided (data not shown).

Figure 9 - Sex of veterinarians who answered the questionnaire in Portugal (*n*= 183).



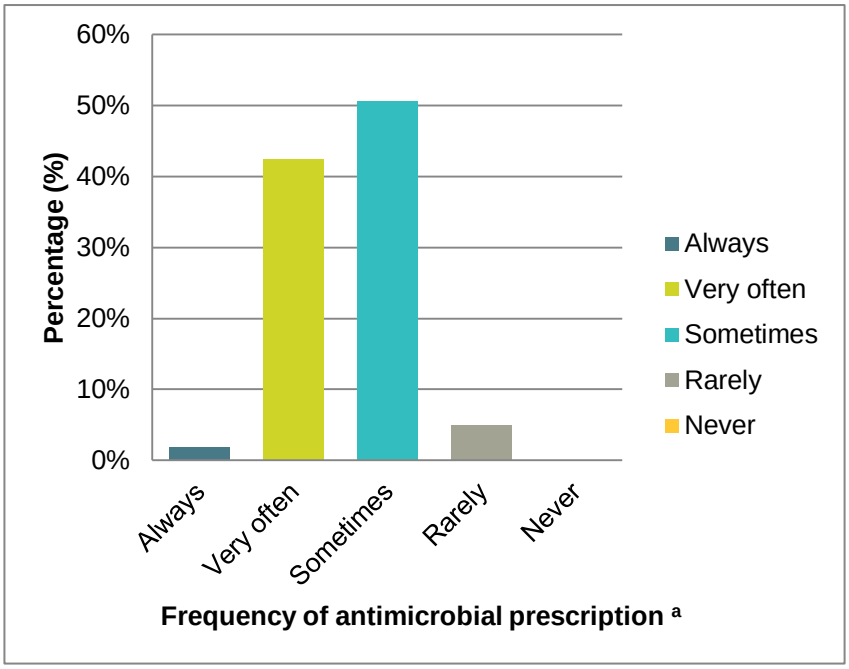
Overall 87% (*n*= 140) of respondents referred the dog as the most common animal species presented for consultation, 11% (*n*= 17) referred to cats and 2% (*n*= 3) referred to other species (Figure 10). From these data further analyses were performed for dogs and cats.

Figure 10 - Distribution of animal species in veterinary practices. Others included rabbits, goats and dogs and cats in the same proportion.



When analysing antimicrobial usage patterns, as illustrated in Figure 11, 2% (*n*= 3) always prescribed antimicrobials on a daily basis, 43% (*n*= 68) prescribed antimicrobials frequently, 51% (*n*= 81) sometimes and 5% (*n*= 8) rarely prescribed antimicrobials on a routine day.

Figure 11 - Frequency of antimicrobial prescription in veterinary practices.
[a] Rarely - One to 5 prescriptions per week; Sometimes – five to ten times per week; very often – more than 10 times per week; Always – a prescription for each appointment.



For dogs and cats the preferred antimicrobial formulations prescribed by veterinarians were oral preparations with 74% (*n*= 119) and 58% (*n*= 92) respectively. Injectable antimicrobial accounted for 25% (*n*= 40), 41% (*n*= 66) and topic solutions were only applied by veterinarians in 1% of animals (both species). The preference of antimicrobial preparation for dogs and cats is presented in Figure 12 and Figure 13 respectively.

Figure 12 - Preference antimicrobial administration route for dogs.

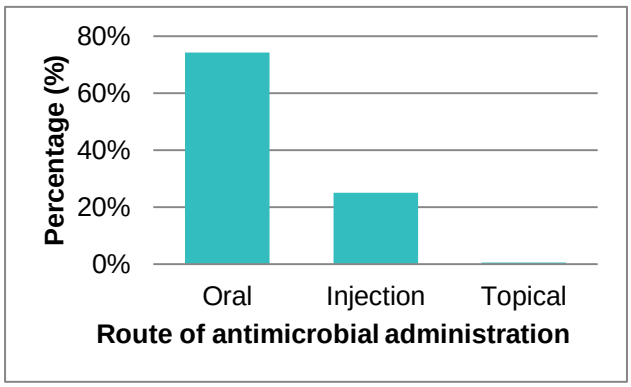
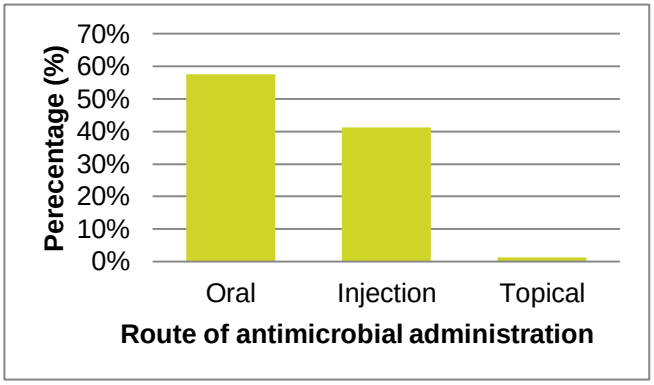
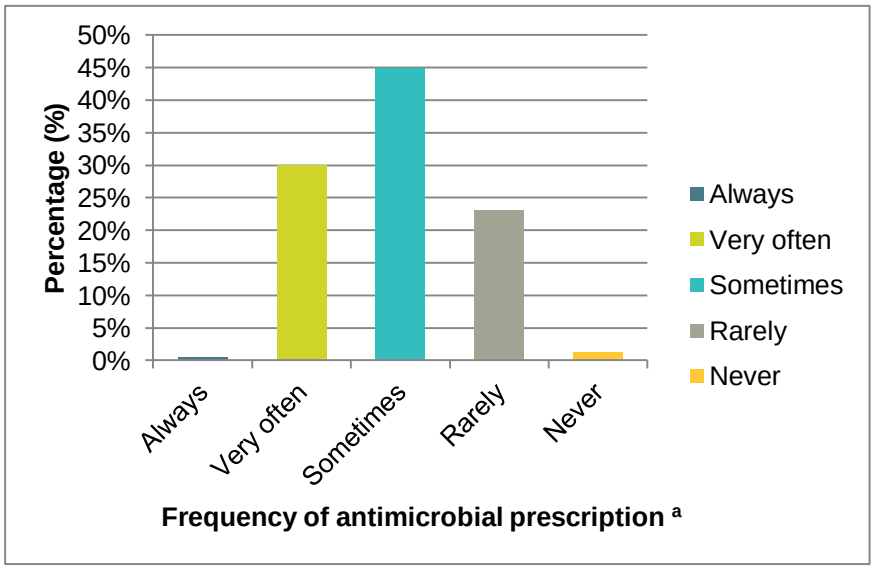


Figure 13 - Preference antimicrobial administration route for cats.



Antimicrobials are prescribed on a normal routine day. To understand the patterns of antimicrobial prescription, it was asked some questions to the veterinarians about their prescription habits, as illustrated in Figure 14. The question: *How often do you prescribe antimicrobials empirically without a confirmed diagnosis?* The answers were: 1% (*n*= 1) always used antimicrobials with no diagnosis made; 30% (*n*= 48) very often prescribed before the diagnosis is made; 45% (*n*= 72) sometimes; 23% (*n*= 37) performed some tests before prescribing and 1% (*n*= 2) never prescribed any antimicrobial without confirmation of the diagnosis.

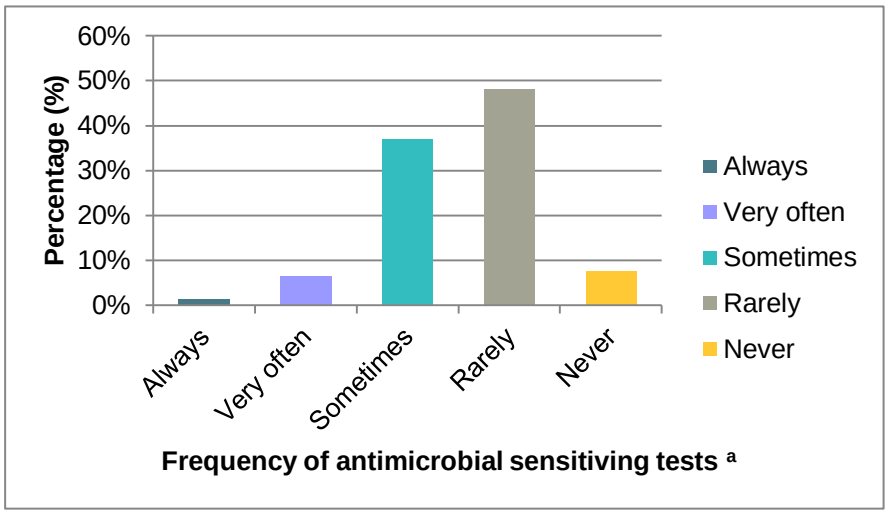
Figure 14 - Frequency of antimicrobial prescription without a diagnosis confirmed.
[a] Rarely - One to 5 prescriptions per week; Sometimes – five to ten times per week; very often – more than 10 times per week; Always – a prescription for each appointment.



In the following of the previous question: *How often do you use culture and antimicrobial susceptibility tests before prescribing antimicrobials*?

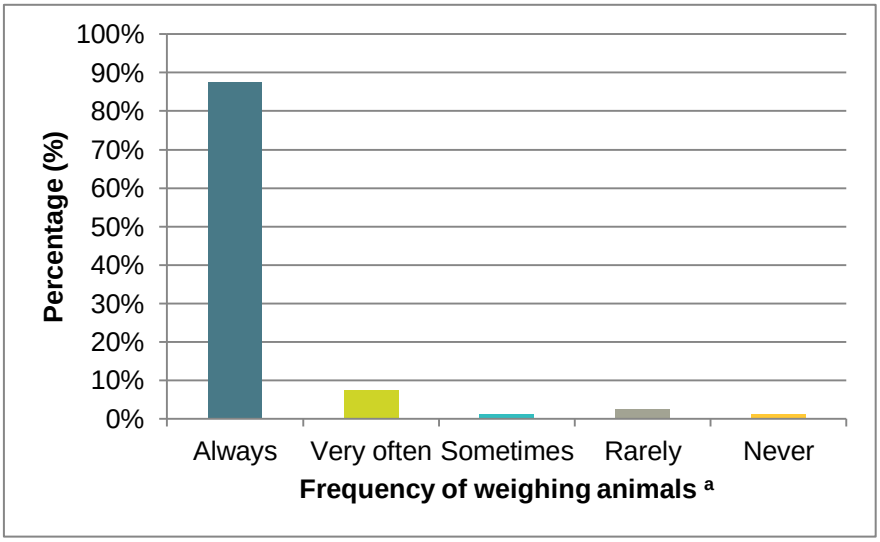
The respondents were consistent with the previous answer. One percent (*n*= 2) always performed tests; 6% (*n*= 10) very often used culture and susceptibility tests, 37% *(n*= 59) sometimes 48% *(n*= 77) rarely performed any test and 8% (*n*= 12) never used any kind of tests before prescribing antimicrobials (Figure 15).

Figure 15 - Frequency of susceptibility testing performed before prescribing antimicrobials. [a] Rarely - One to 5 prescriptions per week; Sometimes – five to ten times per week; very often – more than 10 times per week; Always – a prescription for each appointment.



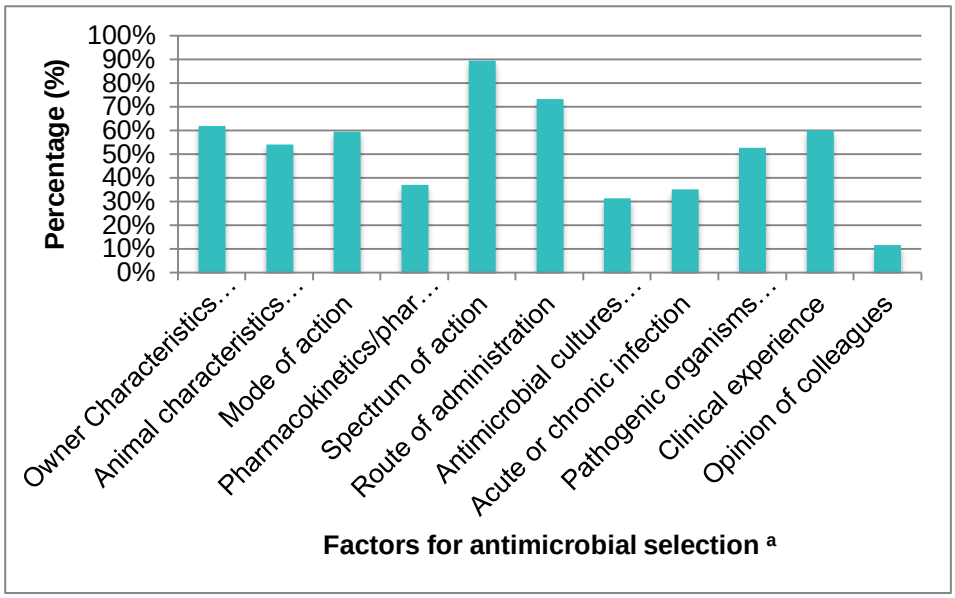
Eighty-eight per cent (*n*= 140) always weighed animals, 8% (n= 12) weighed animals frequently, 1% (*n*= 2) weighed animals sometimes, 3% (*n*= 4) weighed animals rarely, and 1% (*n*= 2) never weighed animals for which they prescribed antimicrobials (Figure 16).

Figure 16 - Frequency of weighing animals for which antimicrobials are prescribed. [a] Rarely - One to 5 prescriptions per week; Sometimes – five to ten times per week; very often – more than 10 times per week.



When asked about the importance of factors when deciding to treat an animal with antimicrobials, spectrum of action (*n*= 143; 89%) was found to be the most important factor, followed by route of administration (*n*= 117; 73%) and owner characteristics (n= 99; 62%) (Figure 17).

Figure 17 - Main factors considered when selecting an antimicrobial for treatment. [a] Owner Characteristics (economics, compliance); Animal characteristics and behaviour; Mode of action; Pharmacokinetics/pharmacodynamics; Spectrum of action; Route of administration; Antimicrobial cultures susceptibility testing; Acute or chronic infection; Pathogenic organisms involved; Clinical experience; Opinion of colleagues.



The most commonly antimicrobial prescribed for dogs using a scale of 1-5 (with 1 being the most frequent, and 5 being the least frequent) was amoxicillin-clavulanate (*n*= 153), followed by enrofloxacin (*n*= 132*)* and metronidazole (*n*= 126) (Figure 18 and Figure 19). For cats amoxicillin-clavulanate (*n*= 151) was also the most important antimicrobial prescribed, followed by enrofloxacin (*n*= 134) and doxycycline (*n*= 120).

Figure 18 – Most common antimicrobial recommended for dogs.

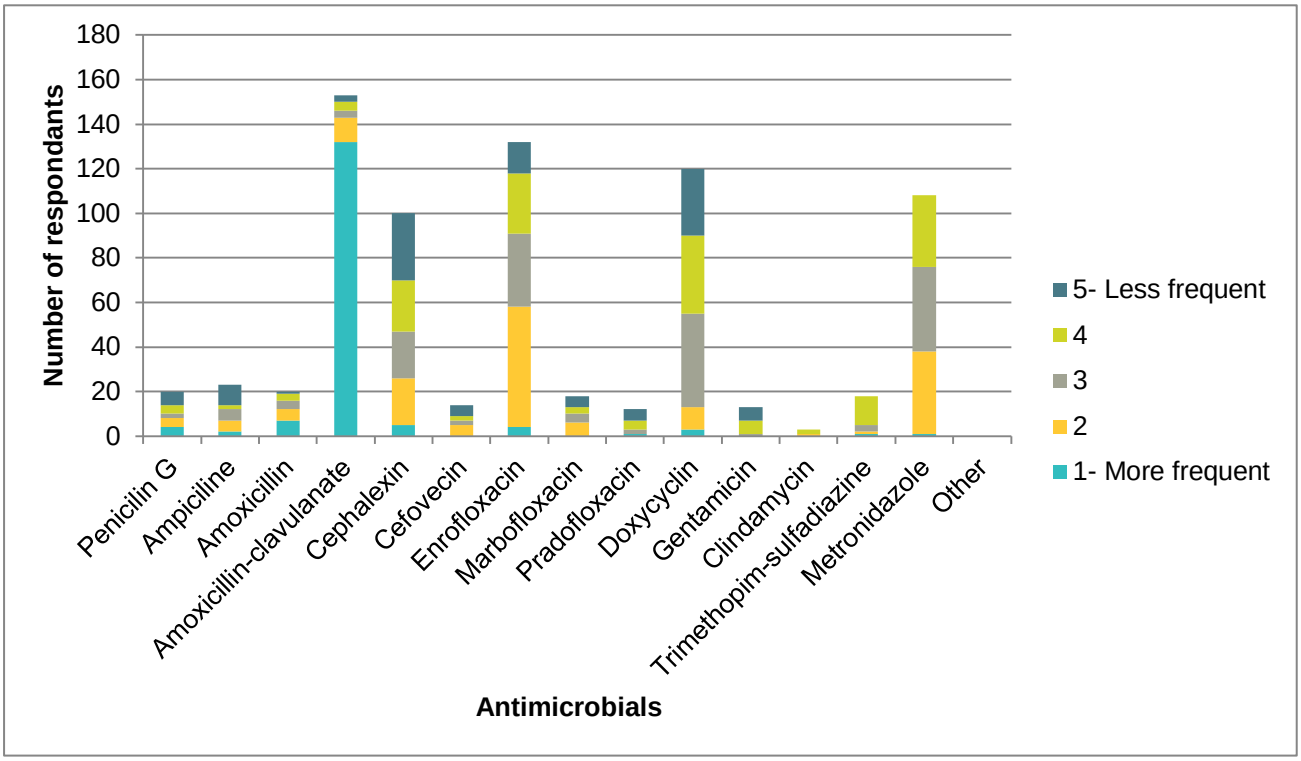
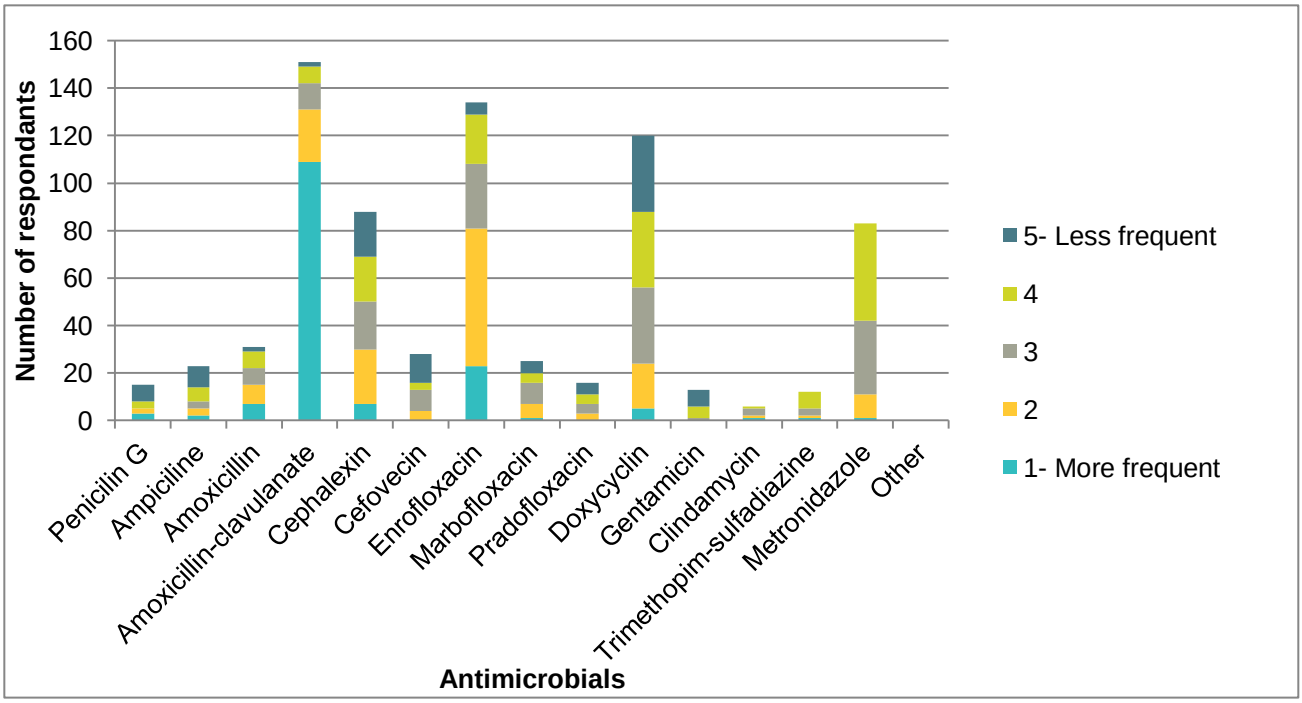


Figure 19 – Most common antimicrobial recommended for cats.



3.3.3. Appropriateness of antimicrobial use

In the last section of the survey (5 questions) the veterinarians were asked to choose among the different antimicrobials, which you would prescribe for each condition. In each question the more common clinical conditions in dogs and cats were included. The total respondents in this section of the survey was equal in all five questions (*n*= 102). Results were analysed by animal species as follows. In annex 4, tables with percentage values for each figure are presented.

For skin/soft tissue disorders, a total of possible prescriptions were (*n*= 965). Overall, the most frequent antimicrobial chosen was amoxicillin-clavulanate (38%), followed by enrofloxacin (18%) cephalexin (15%), and metronidazole (7%) (data not shown).

In dogs, for all conditions presented, amoxicillin-clavulanate was the drug of choice, followed by cephalexin in pyoderma and deep pyoderma, while in bacterial otitis was enrofloxacin (23%). For abscess conditions metronidazole was the second choice of veterinarians (18%) (Figure 20). In cats, amoxicillin-clavulanate was also the antimicrobial of choice followed by enrofloxacin and cephalexin. In abscess, metronidazole (14%) was the third choice (Figure 21).

Figure 20 - Skin disorders in dogs and the antimicrobials chosen by veterinarians (*n*= 102).

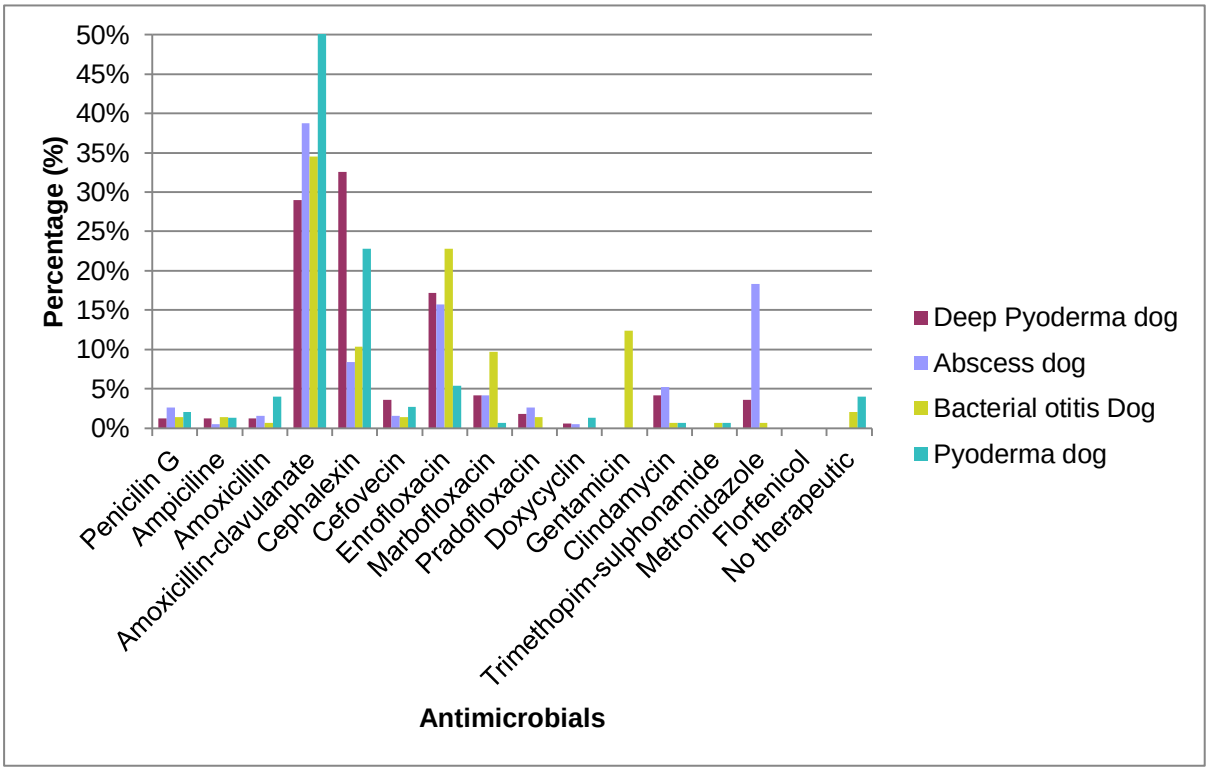
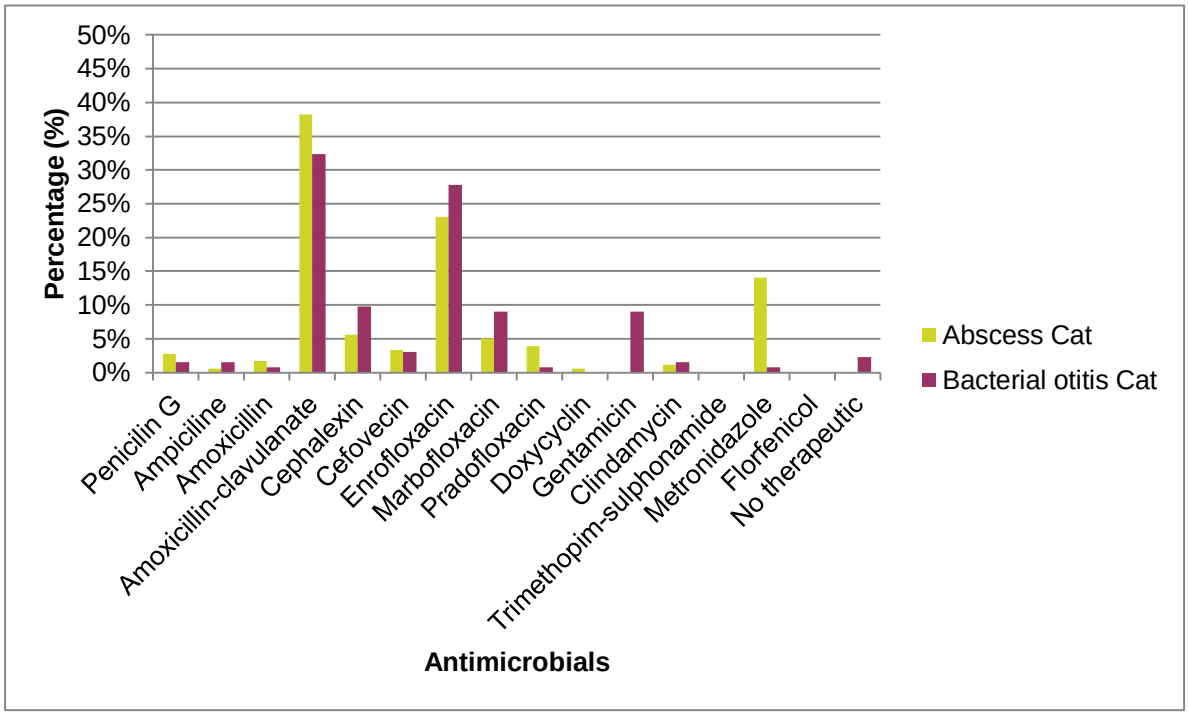


Figure 21 - Skin disorders in cats and the antimicrobials chosen by veterinarians (*n*= 102).



For urinary tract infections (including complicated UTI) enrofloxacin was the most prescribed antimicrobial in cats and dogs followed by amoxicillin-clavulanate and cephalexin (Figure 22 and Figure 23). In pyometra condition in dogs, amoxicillin-clavulanante (34%) was the choice of veterinarians. In cats, FLUTD (a cat specific condition – explained in the discussion of the results), and for 42% veterinarians no antimicrobial would be used for treatment.

Figure 22 - Urinary tract infections disorders in dogs and the antimicrobials chosen by veterinarians (*n*= 102).

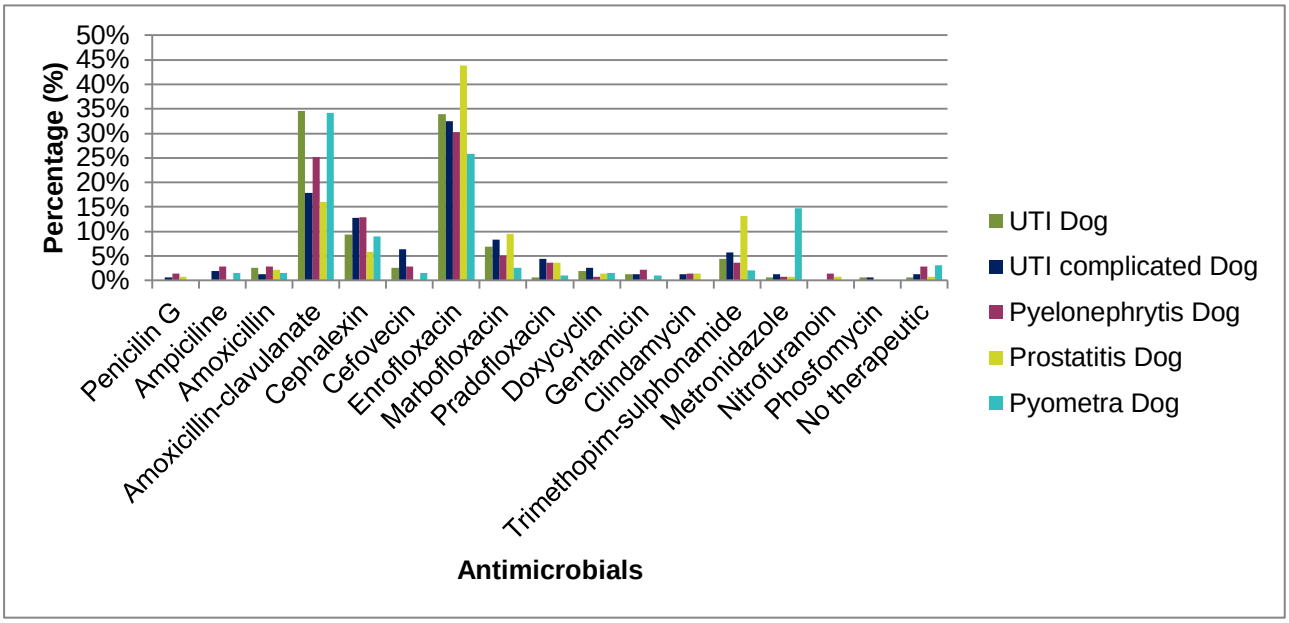
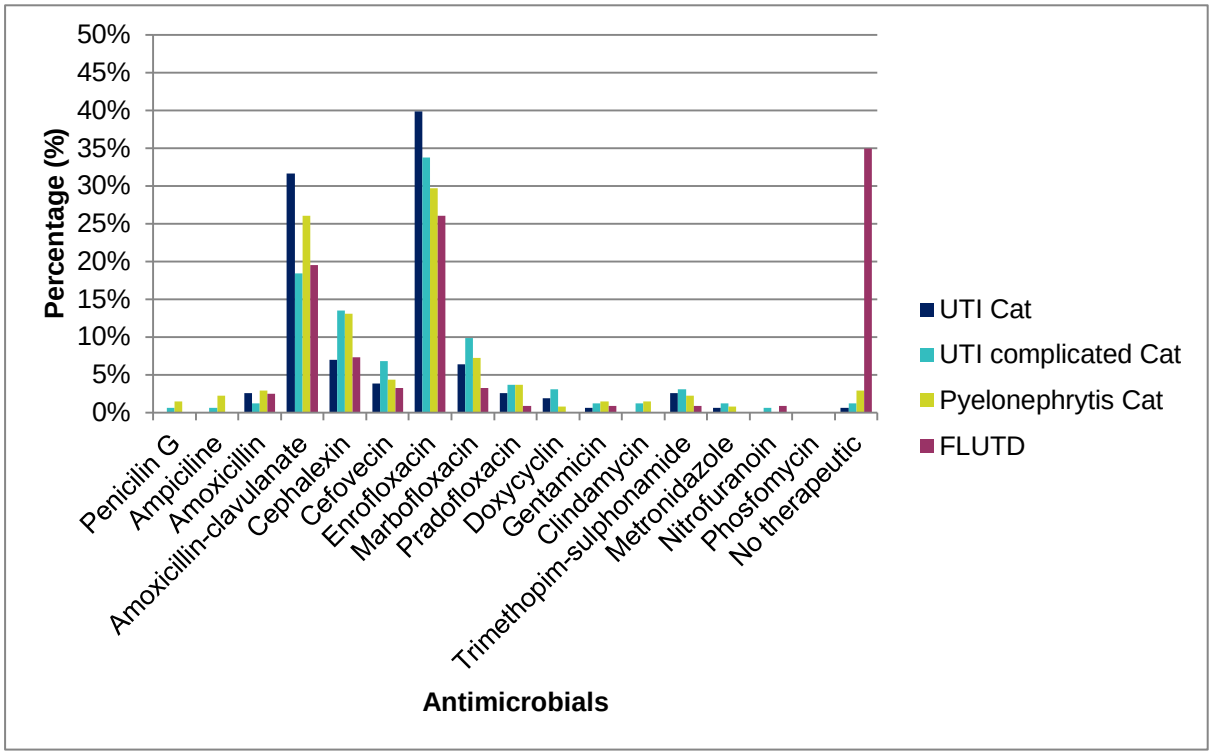


Figure 23 - Urinary tract infections disorders in cats and the antimicrobials chosen by veterinarians (*n*= 102).



In gastrointestinal conditions, in dogs and cats, metronidazole was the antimicrobial of choice followed by amoxicillin-clavulanate and enrofloxacin. For chronic gastroenteritis in both dogs and cats no antimicrobial listed was used, 45% and 47% respectively (Figure 24 and Figure 25).

Figure 24 – Gastrointestinal disorders in dogs and the antimicrobials chosen by veterinarians (*n*= 102).

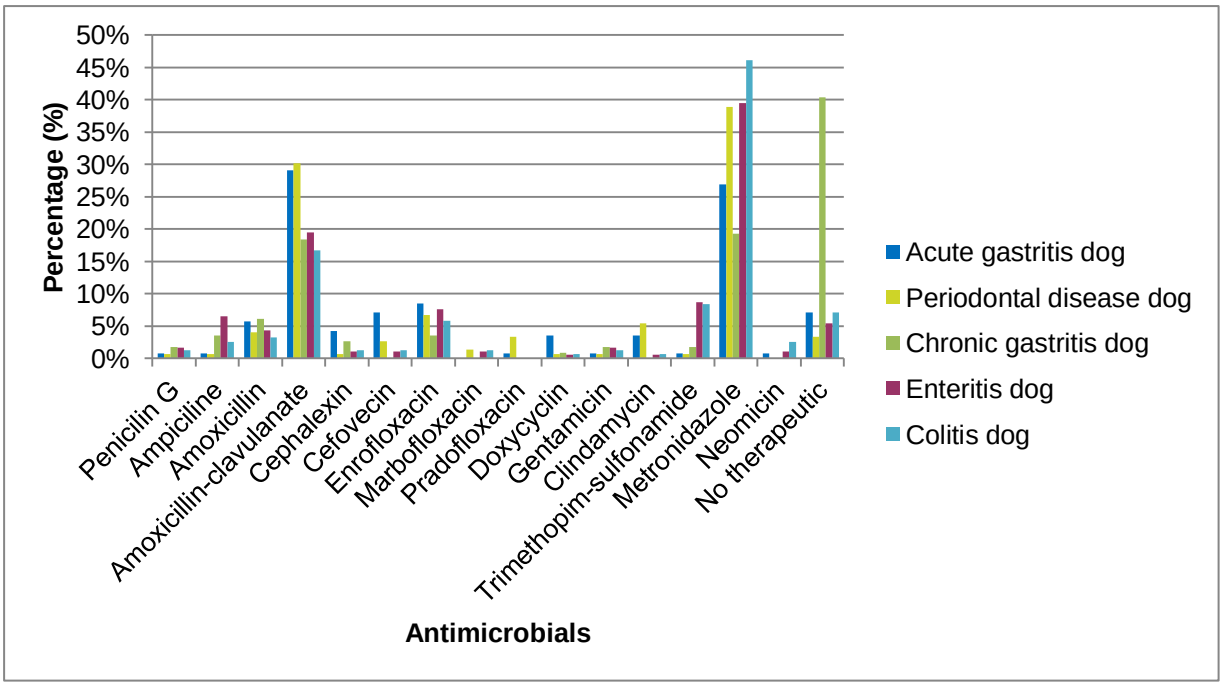
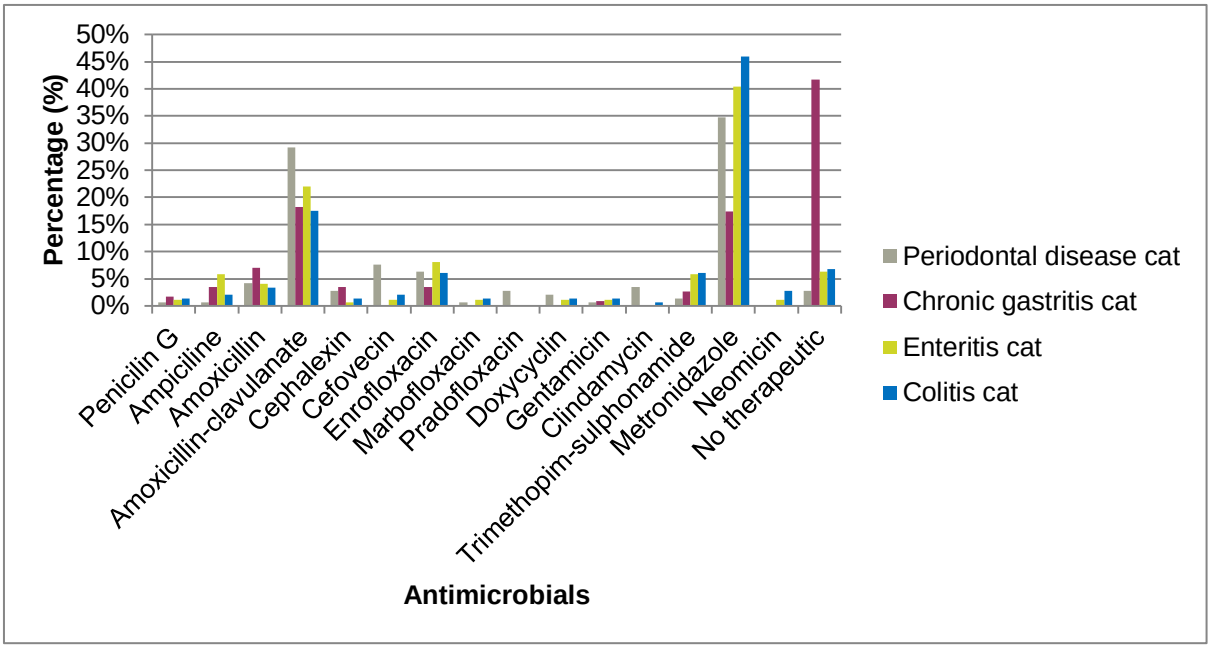


Figure 25 - Gastrointestinal disorders in cats and the antimicrobials chosen by veterinarians (*n*= 102).



For the respiratory tract disorders presented, amoxicillin-clavulanate was the antimicrobial of choice, followed by enrofloxacin, in pneumonia and pyothorax (29%; 25% respectively), in dogs. Doxycycline was the first line preferred by veterinarians for tracheobronchitis (Figure 26). In cats, for feline upper respiratory tract disease and feline respiratory complex, amoxicillin-clavulanate (40%; 32%) was the therapeutic option by veterinarians, followed by doxycycline (21% and 28%). Exception was made for pneumonia and pyothorax for which enrofloxacin was the second choice by veterinarians (Figure 27).

Figure 26 - Respiratory tract conditions in dogs disorders and the antimicrobials chosen by veterinarians (*n*= 102).

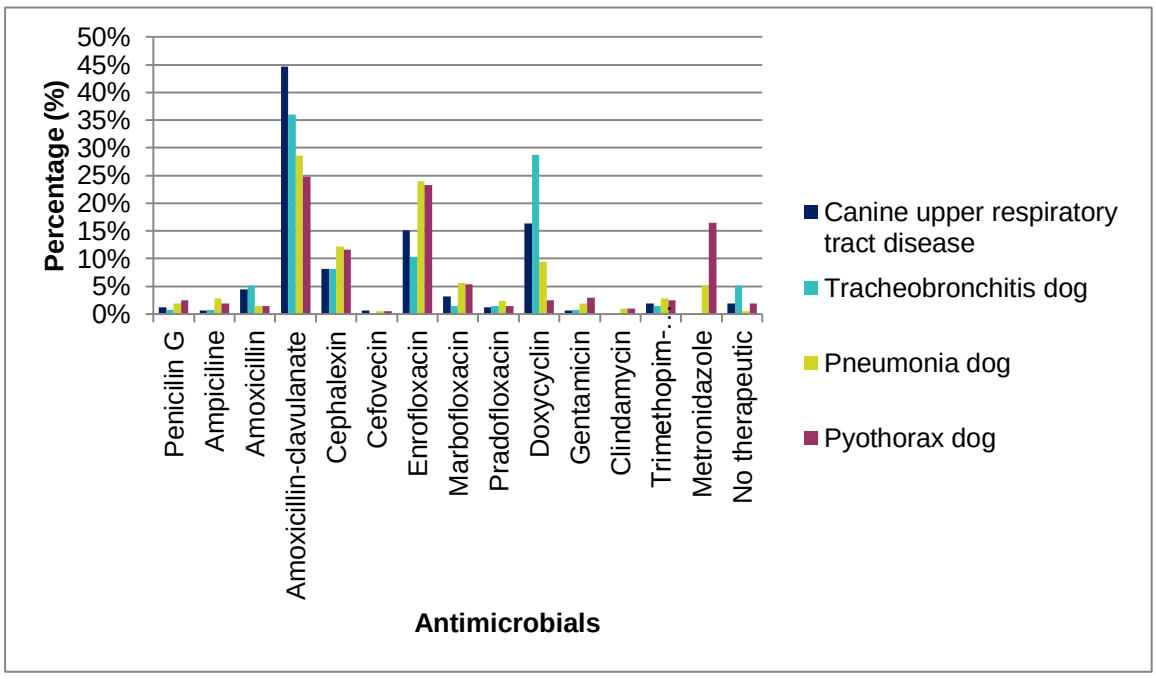
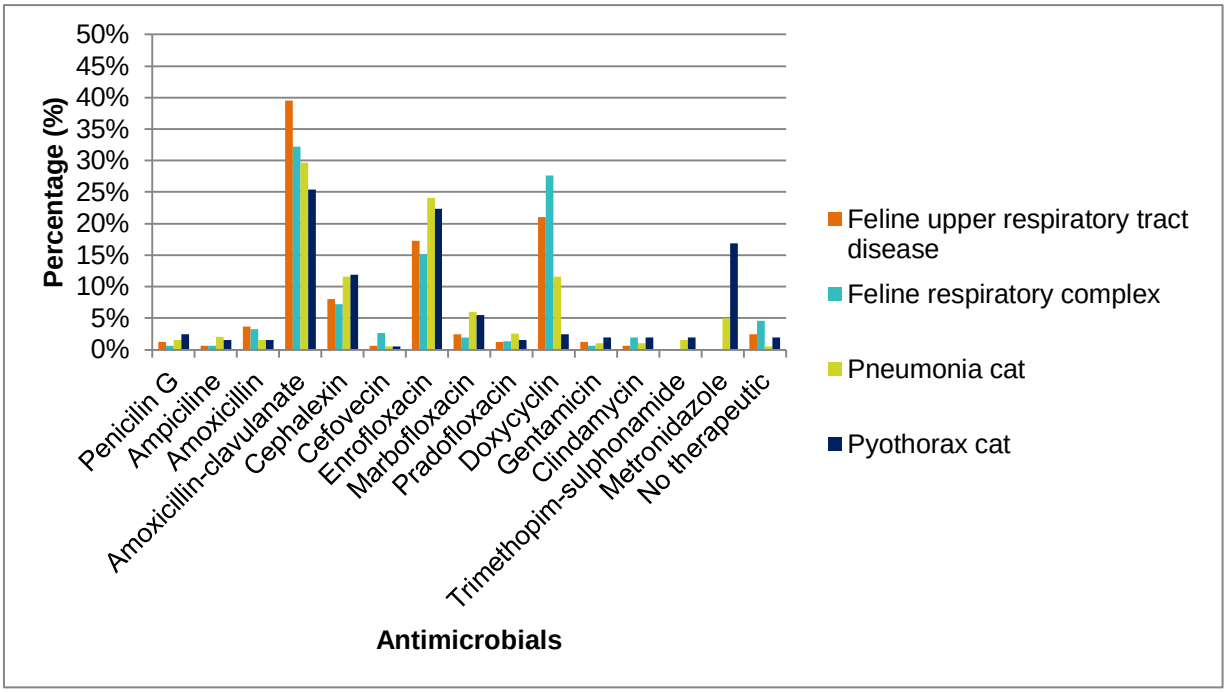


Figure 27 - Respiratory tract infections in cats and the antimicrobials chosen by veterinarians (*n* =102).



Clindamycin was the antimicrobial of choice for osteomyelitis in dogs and cats representing 25% and 22% of veterinary choices respectively; cephalexin was the second option (23%), followed by amoxicillin-clavulanate (14%). In this question we also asked which drugs were prescribed before surgery. Most of the veterinarians selected amoxicillin-clavulanate (42% in dogs and cats), and 19% referred that no therapeutic was used as prophylaxis in both dogs and cats (Figure 28 and Figure 29).

Figure 28 - Orthopedic diseases – prophylaxis in dogs and the antimicrobials chosen by veterinarians (*n*= 102).

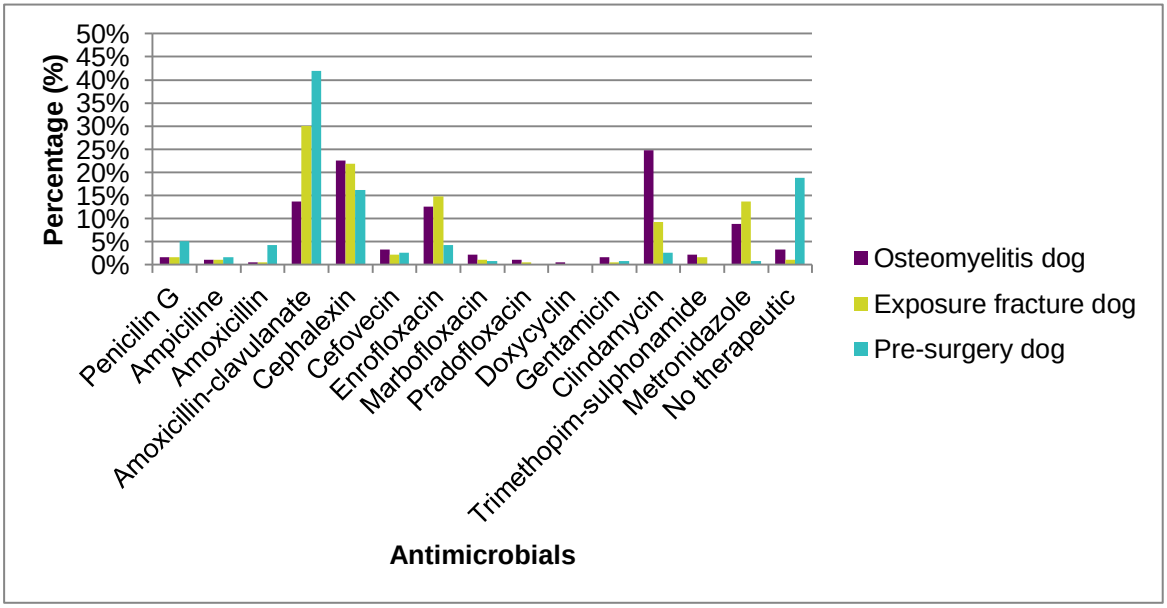
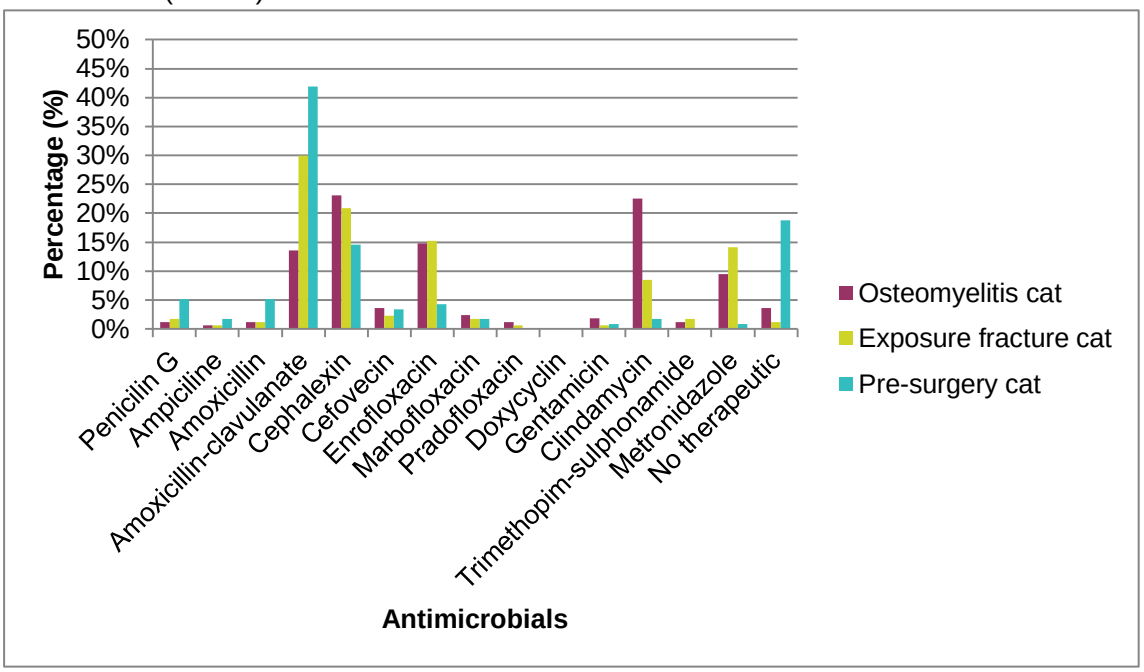


Figure 29 - Orthopedic diseases – prophylaxis in cats and the antimicrobials chosen by veterinarians (*n*= 102).



3.4. Discussion

The increasing public awareness of antimicrobial resistance has led to a network surveillance regarding antimicrobials usage in both veterinary and human medicine. This survey represents a good method for collecting data about antimicrobial prescription patterns in companion animals in Portugal. The response rate in our study was low (3%). However it is considered satisfactory as a pilot study performed in Portugal. This percentage is not consistent with a previous study conducted in the UK where an useable response rate of 51% was confirmed (Hughes *et al*., 2012). According to Chauvin (2002), when a reminder is sent the response rate increases (Chauvin, Beloeil, Orand, Sanders & Madec, 2002). In this study no reminder was sent out because the availability of e-mail addresses was a limiting factor.

The questionnaire about the patterns of antimicrobial prescribing provides an overall view. It is however essential to have a validated questionnaire with a reasonable number of questions, so the response rate may be reasonable and statically representative of the sample. In our study the third part (prescription-based condition antimicrobial usage) was considered for some veterinarians as being long and detailed. This opinion might have influenced the response rate in this part of the questionnaire, since those 81 veterinarians did not complete this last part of the survey. In further studies these issues will be addressed in order to increase the response rate to relevant sections.

The most common animal species attending at the veterinary practice was the dog (87%) whereas the cat represented only 11% according to respondents in this study.

Regarding the prescription of antimicrobials on a normal day at work, according to our study, 43% per cent prescribed very often (more than 10 prescriptions a day) and 51% prescribed sometimes (from 5 to 10 prescriptions a day). These results are in agreement with other studies where is reported that antimicrobials agents were used very often in small animal practice (Grave, Bangen, Engelstad & Soli, 1992).

When asked about the importance of criteria when choosing an antimicrobial, the spectrum of action of an antimicrobial was the most important parameter for the majority of veterinarians (89%), followed by route of administration (73%) and owner characteristics (62%). Interestingly the organism involved in the infection was only ranked in the 6th in the level of relevance (52%). Our results are not in agreement with previous studies. According to Hughes (2012) the main factor in which a veterinarian chooses an antimicrobial was the clinical signs presented, followed by bacterial culture and ease of administration (Hughes *et al*., 2012).

Many veterinary organizations have published guidelines for prudent or judicious antimicrobial use (CVMA, 2000; Morley *et al*., 2005; AVMA, 2006) underlying the importance of performing bacterial culture and susceptibility to confirm diagnosis and to guide while in therapy. However, in this study most veterinarians referred to not performing routinely cultures or susceptibility testing before antimicrobial prescribing. Only 1% of the veterinarians referred that they always performed cultures and susceptibility testing before prescribing an antimicrobial. Forty eight per cent answered that rarely performed these tests, which is consistent with other similar studies (Thomson, Rantala, Viita-Aho, Vainio & Kaartinen, 2009). According to antimicrobial prudent use it is recommended to know which organism is causing the infection to make a correct decision about the antimicrobial to use and to avoid treatment failure (BSAVA, 2013) . Besides that, performing cultures would potentially reduce the number of inappropriate antibiotic prescriptions (Stegemann *et al*., 2006).

In our study most veterinarians (88%) always weighed animals before prescribing antimicrobials. Weighing animals before prescribing an antimicrobial is in agreement with recommendations on antimicrobial use. As a consequence, the veterinary prescribes the correct dose of antimicrobial. Some previous studies *in vitro* have pointed out a relation between exposing bacteria to a sub-inhibitory concentration of antimicrobials and the rise of antimicrobial resistance which leads to a less efficacy in the treatment (Choe, Bouhaouala, Brook, Elliott & Knudson, 2000; Gillespie, Basu, Dickens, O'Sullivan & McHugh, 2005).

In our study the main antimicrobial prescribed for dogs and cats were potentiated amoxicillin and enrofloxacin. The high prevalence of broad spectrum β-lactams is consistent with data previously reported in Finland (Rantala, Hölsö, Lillas, Huovinen & Kaartinen, 2004; Thomson *et al*., 2009). In Australia, amoxicillin-clavulanate and first generation cephalosporins were considered also the most commonly used drugs in dogs (Watson, 2001). The large use of enrofloxacin in dogs and cats is a matter of concern since fluoroquinolones are considered critically important in human medicine. It is therefore important that their use is restricted to complicated clinical situations in order to maintain their effectiveness (Rantala *et al*., 2004). In a study conducted in the United Kingdom, the use of enrofloxacin accounted for 3-4% in dogs and 4-5% in cats Usage of broad spectrum antimicrobials such potentiated amoxicillin and enrofloxacin may be also an indicator of uncertainty or inaccurate diagnosis of the underlying condition (Mateus, Brodbelt, Barber & Stärk, 2011). In Portugal, enrofloxacin is authorized for use in dogs and cats. It is indicated for using in several conditions (e.g. respiratory tract infections, urinary tract infections and skin infections) (Apifarma, 2011). Current guidelines of the British Small Animal Veterinary Association recommends whenever is possible the use of narrow spectrum antimicrobials (BSAVA, 2013). This recommendation

is based on the fact that a broad spectrum antimicrobial should have less impact on the normal microbiota (Hughes *et al*., 2012). However, in some clinical conditions such as pyoderma, pyothorax and periodontitis broad spectrum antimicrobials are recommended (Guardabassi *et al*., 2008; Maddison, 2009). The use of broad spectrum antimicrobials may also indicate the lack of tests performed in order to identify the organism responsible for the infection. The use of fluoroquinolones, a broad spectrum antimicrobial class may reflect the awareness of veterinarians about the β-lactam resistant bacteria or a perception that fluoroquinolones are more suitable in certain clinical scenarios(Hughes *et al*., 2012).

In dogs and cats interestingly the less prescribed antimicrobials were clindamycin, pradofloxacin and cefovecin. Usage of cefovecin was low probably due to the cost involved for treating medium to large dogs. In cats, usage was also very low. According to Mateus (2011) the use of cefovecin in cats was high, which had not been previously reported (Thomson *et al*., 2009; Mateus *et al*., 2011).

In the third part of this survey, the veterinarians were asked to choose an antimicrobial agent for the most common clinical conditions in dogs and cats. Most veterinarians preferred to use clavulanate-amoxicillin for the treatment of all skin infections. This antimicrobial is now recommended for the treatment of pyoderma (Vanni *et al*., 2009). It is also interesting that the use of trimethoprim-sulphonamides, lincosamides and tetracyclines was low. These findings were in agreement with similar studies carried out in Finland and Australia (Watson, 2001; Rantala *et al*., 2004). The low percentage of trimethoprim-sulphonamides in our study is also consistent with other studies where the emergence of resistance in staphylococci is a limiting factor for trimethoprim-sulphonamides usage (Rantala *et al*., 2004). There are also several side effects that have been reported (e.g. thrombocytopenia, leukopenia, crystalluria and fever) (Swedish Veterinary Association [SVA] (2009).This can also be a reason for the low preference by veterinarians. In the case of lincosamides it is now recommended that they should not be used for canine pyoderma treatment (Rantala *et al*., 2004).

In our study the prevalence use of enrofloxacin in dogs and cats for urinary tract infections when compared with previous studies is in disagreement, since urinary tract conditions are mainly treated with β-lactams (Murphy *et al*., 2012). In cats for instance are seldom caused by bacteria but more due to underlying disease causes such as urethra plugs and urinary calculi (Thomson *et al*., 2009). Fluoroquinolones should not be used to treat an uncomplicated UTI unless the culture and susceptibility testing have showed that others antimicrobials are not effective (SVA, 2009)

The term feline lower urinary tract disease (FLUTD) was first used in 1980 to describe variable combinations of straining, hematuria, pollakiuria (frequent passage of small amounts of urine), and periuria (urinations in inappropriate locations (Westropp, 2008). Interestingly, in our study, 43% did not choose any therapeutic for FLUTD, which is according to the guidelines. However, in many occasions it was treated with enrofloxacin (31%) and amoxicillin-clavulanate (34%) consistent with previous reports (Watson, 2001). Since no specific test is used to diagnose FLUTD, the disease remains a diagnosis of exclusion. The frequent use of antimicrobials is concerning since bacteria is not the underlying cause for this condition, it is mainly stress related (Watson, 2001). After the treatment with antimicrobial, some relief is noted, but might be caused by treatment of the underlying cause (Thomson *et al*., 2009). The implementation of MEMO – "Multimodal Environmental Modifications and the increased intake of water are the main recommendations (SVA, 2009).
For pyelonephritis, enrofloxacin and amoxicillin-clavulanate were the preference choice of the veterinarians. According to the Swedish Veterinary Association (SVA) culture and antimicrobial susceptibility tests should be performed before antimicrobial treatment (SVA, 2009). However, renal function remains at risk while pending for the results, empirical treatment should be started as soon as possible (SvHKS, 2013).

Our findings refer metronidazole as the antimicrobial of choice for most of the gastro-intestinal clinical conditions, followed by amoxicillin-clavulanate and enrofloxacin. These findings are in agreement with a study conducted by German (2010), where the most common used antimicrobial were amoxicillin-clavulanate and metronidazole (German, Halladay & Noble, 2010). Also, according to Watson and Maddison (2001), most Australian veterinarians use antibiotics in dogs with unspecific gastroenteritis (Watson, 2001). The use of antimicrobials for gastro-intestinal clinical conditions is not normally considered according to some studies (Rantala *et al*., 2004; Watson, 2001), because the antimicrobials may change the microflora and exert some selective pressure for the emergence of resistant strains of bacteria (Hughes *et al*., 2012).

In addition, amoxicillin-clavulanate was the antimicrobial of choice to treat all respiratory clinical conditions, followed by doxicycline and/or enrofloxacin. These data is consistent with previous studies (Murphy *et al*., 2012). The use of cefovenin in our study was low (1%-4% in both dogs and cats) in all clinical conditions presented. These results are consistent with specific recommendations because this antimicrobial is not approved for respiratory tract infections. However, they are in disagreement with others in which cefovecin was the second most commonly prescribed antimicrobial in cats (Murphy *et al*., 2012). Cefovecin is a third generation cephalosporin with a long term duration (14 days), recommended as a first choice

drug for staphylococci infection when owner compliance and or administration can be a problem (Hughes *et al*., 2012). The use of doxyclicline in our study in most conditions presented is the second choice, except for pneumonia (both in dogs and cats), where amoxicillin-clavulanate was the main choice followed by enrofloxacin.
Doxycycline is a recommended antimicrobial for tick borne infections and canine tracheitis/bronchitis, commonly known as “kennel cough” (SVA, 2009; SvHKS, 2013).

When considering the use of antimicrobials for prophylaxis in many surgical procedures, participating veterinarians referred amoxicillin-clavulanate as the most common antimicrobial chosen (48% in both dogs and cats). Our results are in agreement with the literature where the use of amoxicillin-clavulanate is considered to be more effective and less likely to cause antimicrobial resistance (Rantala *et al*., 2004). According to the American College of Veterinary Internal Medicine´s *Consensus Statement on Antimicrobial Drug Use* notes that prophylactic administration of antibiotics is justified in some cases (Morley *et al*., 2005). In orthopaedic clinical conditions, also amoxicillin-clavulanate was the preference (54% and 52% in dogs and cats respectively), followed by cephalexin with 39% and 36% respectively. In osteomyelitis the antimicrobial choice was clindamycin (44% and 37% in dogs and cats) which is consistent with previous studies (Watson, 2001)

Our findings suggested that the β-lactam antimicrobials class was the most commonly prescribed and these results are similar to those of previous studies investigating antimicrobial use in veterinary practices (Watson, 2001). This fact may be due to the ease administration, few side effects and low cost. However, the use of this drug as a first choice in many clinical conditions without culture and susceptibility testing is not appropriate (AVMA, 2007).

Enrofloxacin was the second antibiotic choice in this study. The frequency of antimicrobial prescription events with fluoroquinolones reported in this study is considered higher than previous reports (Rantala *et al*., 2004; Weese, 2006; Mateus *et al*., 2011). Enrofloxacin is a wide-spectrum, bactericidal antibiotic used for treating mixed infections. The low percentage of cultures performed in this study indicates a probable inappropriate use of enrofloxacin in uncomplicated urinary tract infections Their use should be based on current guidelines specially since enrofloxacin resistance is being associated with multidrug resistance (Weese, 2006; Shea, Annie, Mccarthy & Lindenmayer, 2012). In order to decrease the development of antimicrobial resistance, it is recommended to use narrow spectrum antibiotics whenever possible, so perhaps in some situations an alternative choice of antibiotic would have been more prudent (AVMA, 2007).

In this study neomicin (*n*= 13) was chosen for colitis and enteritis in dog and cats; nitrofurantoin (*n*= 5) for complicated urinary tract infections in cats (*n*= 1), pyelonephritis in dogs (*n*= 2), prostatitis in dogs (*n*= 1) and FLUTD (*n*= 1); and phosphomycin for treating uncomplicated and complicated urinary tract infection in dogs (*n*= 2).

As mentioned previously, cats and dogs were exposed to some critically important antimicrobials in veterinary and human medicine. In this study, the low response of veterinarians to answer the third section of this survey was a limiting factor for understanding the patterns of antimicrobial prescription. The results presented cannot determine whether the usage of antimicrobials in dogs and cats is adequate. Therefore further studies and a continuous surveillance system for antimicrobial usage with more detailed information is necessary to evaluate the risk of antimicrobial resistance in companion animals. To analyse and conclude about the usage of antimicrobials in companion animals, complete and accurate data is needed addressing the animal species, such as, age, weight, clinical condition. Other aspects as antimicrobial agent, frequency, route of administration, duration of the treatment and previous antimicrobials prescribed are crucial to understand the patterns of prescription and evaluate the risk of antimicrobial resistance, and assess if guidelines are being followed by veterinarians. In our study it was not possible to gather all this important data since the compliance was not high in this survey. This reflects the importance of gathering clinical and antimicrobial usage data from electronic patient management systems.

Part 2

Study 2

4. Characterization of cephamycin resistant in Enterobacteriaceae

4.1. Aim of this study

The resistance to β-lactams producing Enterobacteriaceae is a public health issue. The genes encoding β-lactamases may be present in transferable plasmids or in the chromosome. Plasmids play an important role in the horizontal transfer and there is a potential zoonotic risk of dissemination of these resistant genes between humans and animals. The information about the plasmid-mediated AmpC β-lactamases among Enterobacteriaceae in companion animals was limited. The objective of this study was to assess the presence of pAmpC genes among Enterobactereaceae isolates collected from companion animals and human clinical isolates. And, subsequently characterize the positive isolates by using molecular methods for plasmid characterization. Identification of pAmpC in Enterobacteriaceae isolated from companion animals and humans may be an evidence of selection pressure arising from both human and veterinary use of β- lactams.

The specific aims of the study were:

(iii) To assess the prevalence of pAmpC β-lactamases producing Enterobacteriaceae in companion animals from Portugal and Denmark;

(iv) To characterize pAmpC β-lactamases producing isolates and compare them, in order to investigate the predominant plasmid types found in Portugal and Denmark respectively.

4.2. Material and Methods

4.2.1. Bacterial strains/isolates

In this study, isolates were collected from Portugal (animal and human origin isolates) and Denmark (only animal origin isolates) (Table 5). The isolates chosen to be included in this study were selected based on their resistance profile. The Portuguese isolates inclusion criteria were: cefoxitin, amoxicillin-clavulanate resistant and cefepime susceptible. A total of 61 (*n*= 61) isolates from animal origin were included (22 *E. coli* isolates, 18 *Enterobacter* spp. isolates, 10 *Proteus* spp. isolates, 9 Klebsiella spp. isolates, 1 *Serratia marsecens* isolate and 1 *Citrobacter freundi* isolate. The isolates includes 41 from urine (67%) 5 from wounds (8%), 5 from skin swab (8%), 4 from ear (7%), 3 from swab (5%), 1 from abdominal fluid (2%) and 1 from prostate liquid and puncture disc (2%). These isolates belonged to the collection in the Laboratory of Antibiotic Resistance from the Faculty of Veterinary Medicine, and were collected from January 2000 to November 2012. Regarding humans isolates, 11 (*n*= 11) *E. coli* isolates belonged to the collection of TechnoPhage, from the Molecular Medicine Institute, Lisbon. The isolates were human community-acquired urinary tract infections from the Lisbon area. Sixteen (*n*= 16) *E. coli* isolates from Denmark, from dogs and cats, belonged to the Veterinary Hospital in Copenhagen, collected from 2010 to October 2012, were included in this study. The inclusion criteria for the Danish isolates were: cefoxitin and amoxicillin-clavulanate resistant. The 16 *E. coli* isolates included 4 from urine (25%), 7 from wounds (44%), 3 were unknown (19%), 1 from ear (6%), 1 from biopsy (6%). All isolates were stored at -80°C. In total, this study started with 88 Enterobacteriaceae isolates and this data was compiled on Excel file for further analysis.

Table 5 - Origin from the isolates included in this study.

	Animal isolates (*n*= 77)				Human isolates (*n*= 11)	
	Portugal (*n*= 61)		Denmark (*n*= 16)		Portugal (*n*= 11)	Total (*n*= 88)
Organism	Dog	Cat	Dog	Cat	Human	
E. coli	18	4	15	1	11	49
Proteus spp.	8	2	-	-	-	10
Enterobacter spp.	11	7	-	-	-	18
Klebsiella spp.	7	2	-	-	-	9
Serratia spp.	1	-	-	-	-	1
Citrobacter spp.	1	-	-	-	-	1
Total	46	15	15	1	11	88

4.2.2. Antimicrobial susceptibility testing

4.2.2.1. The disc diffusion method

Antimicrobial susceptibility testing was performed using the standard disc diffusion method on Mueller-Hinton Agar (MH2, Biomérieux) with an inoculum equal to 0,5 McFarland turbidity, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for animals. The plates were incubated at 37ºC for approximately 18 hours. The following discs (Oxoid) were used: Amoxicillin-clavulanic acid (30µg); Cefoxitin (30µg), Cefotaxime (30µg), Imipenem (30µg), Ciprofloxacin (5µg), Tetracycline (30µg), Gentamicin (10µg), and Trimethoprim-sulfamethoxazole (25µg) (Table 6).

The detection of ESBL producing Enterobacteriaceae was accomplished by the double disc synergy between amoxicillin-clavulanic acid and an expanded-spectrum cephalosporin, for example cefotaxime and/or ceftazidime. The test is considered positive if after the incubation period the inhibition zone between the discs is enhanced (Rupp & Fey, 2003).

The results were registered by measuring the zone of inhibition diameter and interpreted according to the CLSI breakpoints for animals (CLSI, 2008) and according to the human CLSI breakpoints (CLSI, 2012) when the data was unavailable.

Table 6 - Diffusion disc method. Susceptibility criteria according to the Clinical Laboratory Standards Institute.

Antibiotic class	Antibiotic	Abbreviation	Disc (µg)	Diameter [a] S	I	R
Penicillin associations	Amoxicillin/clavulanic acid [b]	AMC	20/10	≥18	14-17	≤13
Cephalosporins	Cefoxitin [c]	FOX	30	≥22	15-21	≤14
	Cefotaxime [c]	CTX	30	≥23	15-22	≤14
Fluoroquinolones	Ciprofloxacin [c]	CIP	5	≥17	14-16	≤13
Aminoglycosides	Gentamicin [b]	CN	10	≥16	13-15	≤12
Tetracycline	Tetracycline [b]	TE	30	≥19	15-18	≤14
Potentiated sulphonamides	Trimethoprim-sulphamethazol[b]	SXT	25	≥16	11-15	≤10
Carbapenems	Imipenem [b]	IPM	30	≥16	14-15	≤13

[a] Inhibition zone diameters were interpreted according to: [(b)] CLSI M31-A3 and [(c)] CLSI M100-S22;
S - Susceptible; I - Intermedius; R - Resistant;

4.2.2.2. Broth micro dilution method

The minimum Inhibitory Concentrations (MIC) of cefoxitin (512 µg/ml), was determined according to CLSI guidelines by broth microdilution for *ampC* β-lactamase producers. *Escherichia coli* ATCC25922 strain was used as control strain for determination of MIC values. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic that completely inhibits growth (Yan *et al*., 2002). The determination of the MIC is considered a gold standard to assess the antibiotic potency, despite its limitations, it should

be performed to validate the results obtained by other methods (MacGowan & Wise, 2001). The results were analysed according to the principle of the MIC, and bacterial growth was easily detected, mostly as a pellet on the bottom of the well.

VetMIC Stördjur® (National Veterinary Institute, Uppsala, Sweden) for small animals was also performed according to the manufacturer´s instructions, to confirm the results obtained by the MIC for cefoxitin. The antibiotics tested were: ampicillin (2 - 8 µg/mL), cephalotin (4 - 16 µg/mL), cefotaxime (0,03 - 12 µg/mL), amoxicillin-clavulanate (2/1 - 8/4 µg/mL), nitrofurantoin (4 - 16 µg/mL), gentamicin (0,25 - 1 µg/mL), trimethoprim-sulphonamide (0,5/9,5 µg/mL), tetracycline (0,5 - 2 µg/mL) and enrofloxacin (0,008 - 0,03 µg/mL). The results were interpreted according to M31-A3 (CLSI, 2008), and M100-S22 (CLSI, 2012) (Table 7).

Table 7 - VetMIC® interpretative criteria for Enterobacteriaceae.

Antimicrobial	Clinical breakpoints					
	M31-A3 (CLSI, 2008) MIC (µg/mL)			M100-S22 (CLSI, 2012) MIC (µg/mL)		
	S	I	R	S	I	R
Ampicillin	≤8	16	≥32	-	-	-
Cephalotin	≤8	16	≥32	-	-	-
Cefotaxime	-	-	-	≤1	2	≥4
Amoxicillin/clavulanic acid	≤8/4	16/8	≥32/16	-	-	-
Nitrofurantoin	-	-	-	≤32	64	≥128
Gentamicin	≤2	4	≥8	-	-	-
Trimethoprim-sulphonamide	≤2/38	-	≥4/76	-	-	-
Tetracycline	≤4	8	≥16	-	-	-
Enrofloxacin	≤0.5	1-2	≥4	-	-	-

4.2.3. Detection and sequencing of β-lactamase genes

4.2.3.1. Total DNA isolation

The protocol to extract DNA was based on the boiling lysis method. Briefly, in a sterile eppendorf®, a loop (disposable - white; 1µl) with 3-4 isolated colony (plated on blood agar and incubated for 18 hours) was dissolved in 100 µl MiliQ water and this suspension was boiled for 10 minutes. Lysate prepares were clarified by centrifugation at 1400 x *g*, 4ºC during 15 minutes. Then the clarified supernatant was transferred into a 1.5ml eppendorf® tube and stored at -20ºC (Féria, 2001).

4.2.3.2. *pAmpC* gene detection

All isolates that exhibited resistance to cefoxitin, after growth from the stored cultures, were screened for the presence of *ampC* genes by the multiplex polymerase chain reaction (PCR) described by Pérez-Pérez & Hanson (2002) *E. coli* J53 pMG144 carrying bla_{DHA-1}, *Proteus mirabilis* 1089/07 carrying bla_{CMY-2}, *E. coli* J53 pMG251 carrying bla_{ACT-1}, *E. coli* J53 PMG 252 carrying bla_{FOX}, Klebsiella Nu2936 carrying bla_{MOX} and *Klebsiella* 96D carrying bla_{MIR} were used as positive control strains.. Isolates positive for the AmpC phylogenetic group CIT were subjected to a specific PCR targeting the entire bla_{CMY} gene. The multiplex PCR was performed with modifications from the described by Pérez & Hanson (2002) (Pérez-Pérez & Hanson, 2002) with a final volume of 50 µl. The primers used for amplification are listed in Table 8. Each reaction contained 1.74 µl of MiliQ water, 3 µM of each of the following primers (10 µM) MOXMF, MOXMR, CITMF, CITMR, DHAMF, DHAMR; 2.5 µM primers ACCMF, ACCMR, EBCMF, EBCMR, FOXMF. FOXMR, 10 µl of 5x PCR buffer without MgCl2; 3 µl of Mgcl2 (25µM), 0.5 deoxyribonucleotide triphosphates (dNTPs) (25 mM), 0,26 µl DMSO 5%, and 0.5 µl of NZYTech Taq (NZYTech, Lisbon, Portugal) The template DNA was added to 48 µl of the master mixture. Thermo cycling conditions included an initial denaturation step at 94˚ C for 3 minutes, followed by 25 cycles of DNA denaturation at 94°C for 30s, primer annealing at 65°C for 30s, primer extension at 72°C for 1 min, and the final step, a single final extension at 72°C for 7 min was added.

PCR products were subjected to electrophoresis on 1,5% agarose gel (NZYTech, Lisbon, Portugal) with tris-borate-EDTA (TBE) 0.5x or tris-acetate-EDTA (TAE) 1x and stained with ethidium bromide 10mg/ml (Invitrogen, CA, EUA), to identify the amplified DNA fragments (as referred below in the protocol). Molecular weight of the PCR amplicons was determined by comparison with a 100-bp ladder molecular weight standard (Ladder V, 100-1000pb NZYTech, Lisbon, Portugal). The bands were visualized by using UV transullimination (Thermal Imaging System FTI-500, Amersham Pharmacia Biotech, Piscataway, EUA).

4.2.3.3 Purification and sequencing of DNA

The PCR products were purified using PCR Product Purification Kit (NZYtech, Lisbon, Portugal) according to the manufacturer's instructions. PCR primers used are listed in Table 8.

4.2.3.4. Data analysis of DNA sequences

DNA sequencing was performed by *STAB VIDA* – DNA sequencing (Oeiras, Portugal). The nucleotide sequences were analysed and compared with known sequences using the basic local alignment search tool (BLAST) which is available on the homepage of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 8 - PCR and sequencing primers used in this study.

Primers	Nucleotide sequence (5´-3´as synthetized)	Application	Expected amplicon size (bp)
Multiplex AmpC			
MOX	F-GCT GCT CAA GGA GCA CAG GAT R-CAC ATT GAC ATA GGT GTG GTG C	PCR	520
CIT	F-TGG CCA GAA CTG ACA GGC AAA R-TTT CTC CTG AAC GTG GCT GGC	PCR	462
DHA	F- AAC TTT CAC AGG TGT GCT GGG T R- CCG TAC GCA TAC TGG CTT TGC	PCR/ sequencing	405
ACC	F- AAC AGC CTC AGC AGC CGG TTA R- TTC GCC GCA ATC ATC CCT AGC	PCR	346
EBC	F- TCG GTA AAG CCG ATG TTG CGG R- CTT CCA CTG CGG CTG CCA GTT	PCR	302
FOX	F- AAC ATG GGG TAT CAG GGA GATG R- CAA AGC GCG TAA CCG GAT TGG	PCR	190
CMY	F- ATG ATG AAA AAA TCG TTA TGC R- TTA TTG YAG CTT TTC AAG AAT GC	PCR/ sequencing	1146
*bla*TEM	F- TACGATACGGGAGGGCTTAC R- TTCCTGTTTTTGCTCACCCA	PCR	716
*bla*SHV	F- TCAGCGAAAAACACCTTG R- TCCCGCAGATAAATCACCA	PCR	471
*bla*OXA	F- TATCTACAGCAGCGCCAGTG R- CGCATCAAATGCCATAAGTG	PCR	199
*bla*CTX-M	F- TTTGCGATGTGCCAGTACCAGTAA R- CGATATCGTTGGTGGTGCCATA	PCR/ sequencing	544

4.2.3.5. Detection of Extended spectrum β-lactamases using PCR multiplex

AmpC β-lactamase producers were also screened by PCR multiplex to detect the presence of TEM, SHV and OXA-1 enzymes, as described by Pomba (2006). CTX-M enzymes were screened by a simplex PCR adapted from Edelstein, Pimkin, Palagin, Edelstein & Stratchounski (2003); Pomba (2006).

4.2.3.6. Plasmid analysis

Plasmid DNA was extracted from all strains producing β-lactamases. The protocol used was adapted from Bimboim & Doly (1979). The protocol followed was: the strains were grown in 10 ml of BHIB (Brain Heart Infusion Broth) overnight. A volume of culture (1.5ml) was transferred to an eppendorf® tube (0.5-1.5 ml) and was centrifuged (13.000 rpm for 2 minutes), and the supernatant was discarded. After, 150 µL SET-buffer (saccharose, EDTA and trisaminometano-TRIS base) was added and vortex immediately. The tubes were centrifuged for 15 seconds. Therefore 350 µl of lysis solution (1% sodium dodecyl sulfate – SDS; 0,2M sodium hydroxide - NaOH) was added and the tubes were gently inverted 10 times. The tubes were maintained for 5 minutes at room temperature and afterwards 250 µl of KAc (potassium acetate) solution was added. The tubes were maintained at 0°C for 60

minutes to allow moist of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugations were done for 15 minutes to achieve a clear supernatant. The supernatant was removed and transferred to a new eppendorf® containing 500 µl of isopropanol and the tubes were inverted 10 times. The next step included harvesting the DNA by centrifugation for 15 minutes and discarding the supernatant. The pellet refers to the pDNA which was dissolved in MiliQ water.

4.2.3.7. Transfer of resistance

pDNA was transformed into electrocompetent Genehog® *E. coli* (Invitrogen, Taastrup, Denmark). The protocol followed was: 1µl of pDNA was added to Genehog® *E. coli* and mixed gently with a pipette. Further, was placed in an electroporation cuvette (electrode gap 0.2 cm) and transformed (pulse conditions: 2.5 Kv, 25 µF, 200 Ω). Immediately 1 ml of Brain Heart Infusion Broth (BHIB, Oxoid A/S, Greve, Denmark) pre-warmed at 37ºC was added to the cuvette to wash the cells and transferred to a new eppendorf® tube. This solution was incubated for 1 hour at 37ºC on a heating block to allow expression of the antibiotic resistant gene. Then, 100 µl of this solution was distributed on selective plates and non-selective plates to ensure well-spaced colonies for counting and picking. The plates and the recovery solution were incubated overnight at 37ºC. Transformants were selected on Brain Heart Infusion agar (BHIA, Oxoid A/S, Greve, Denmark) plates containing 2 µg/mL cefotaxime. Selected colonies were analysed by PCR to confirm that the transformants contained the bla_{CMY-2} or the bla_{DHA-1} gene that was found in the donor strain.

4.2.3.8. Plasmid replicon typing

Total DNA from transformants were obtained as described previously using a boiling lysis method and used as a template in a Polymerase Chain Reaction-based Replicon Typing Method (PBRT) using a PBRT-kit (Diatheva, Italy), according to the manufacturer´s instructions. In order to identify the more common replicon circulating among Enterobacteriacea, a method by Carattoli (2005) was developed The plasmid characterization is based on incompatibility (inc) groups (Carattoli *et al*., 2005a).

Plasmids that were negative in the PBRT analysis were designated “non-typeable”. The primers and protocols for the specific incompatibility groups can be retrieved at http://pubmlst.org/plasmid/. The pMLST method is a specific method for plasmid typing based on a DNA sequence which recognizes similar plasmids and their detection among isolates from different countries. This typing method contributes to gather knowledge about the types of plasmids circulating in animals and humans by describing the spread of virulence and resistance plasmids (García-Fernández *et al*., 2008).

Briefly, the pMLST method involved PCR amplifying and sequencing genes essential for replication and maintenance, which include: *pill*, a gene within the pil locus involved in pilus biogenesis; *sogS*, a primase involved in discontinuous plasmid DNA replication; *ardA*, encodes a type-I restriction enzyme, *repI1*, the RNAI antisense regulating system in the IncI1 replication system and the intragenic *trbA*- pndC region involved in maintenance and plasmid transfer respectively (García-Fernández *et al*., 2008).

4.2.3.9. S1 nuclease PFGE

The DNA fragments larger than 25 kb to be separated, it is mandatory to use pulsed-field gel electrophoresis (PFGE), which involves the application of alternating current, from different directions (Boultwood, 1994). According to this principle DNA fragments are separated by the speed at which they reorient themselves with the changes in current direction (Lai, Birren, Clark, Hood & Simon,1989). In order to investigate the presence of plasmids and their size PFGE was performed according to CDC PulseNet protocol (Ribot *et al*., 2006) and is presented below. Both enzymes and buffers used in the restriction enzyme process belonged to Thermo Fisher Scientific Inc., USA. A *Salmonella* serotype B*raenderup* strain (H9812) was used as a reference strain (Hunter *et al*., 2005). Thiourea (100µM) was added to the running buffer. Pictures of PFGE gels were taken by Gel/ChemiDoc system (Bio-Rad Laboratories). The following protocols were used: A pure culture of each isolate is grown in blood agar plates at 37ºC for 18 hours. Cells were suspended in CSB (3 ml) and adjusted to an optical density of 0.83 to 0.87 at 600 nm. Ten µl of proteinase K solution (20mg/ml) was added to optimize cell lysis. Meanwhile the agarose gel solution 1% (SeaKem® Gold agarose, FCM Bioproducts) was prepared and a volume of 200 µl melted agarose was added. Gel plugs were formed by pipetting 200 µl volumes into plug molds and were allowed to solidify at 4°C for 10 minutes. The plugs were transferred to 50 ml tubes containing 5 ml of cell lysis buffer (50 mM Tris-HCl, 50 M EDTA, pH 8.0, 1% N- lauroyl sarcosine, 20 mg/ml proteinase K). The plugs were removed and placed into a 50 ml screw cap tube and were incubated at 54ºC for 2 hours; Plugs were then washed with 10-15 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) twice and with sterile double distilled preheated water (4 times) with agitation at 50º. Plugs were stored in 10 mM Tris-HCl-100 mM EDTA (pH 8.0) at 4°C.

Restriction enzyme digestion

Before running a PFGE, thin slices of a plug of each isolate to be tested was treated with S1-nuclease to linearize the plasmids. For *Salmonella braenderup* (used as a marker) and clinical isolates there was a pre-incubation for 15m at 54ºc with the respective buffer and water. Then, the solution was discarded and restriction with XBA1 (20U/µl) for *S. braenderup* and S1 (100U/µl,) enzyme for the remaining plugs during 45 minutes and two hours

respectively was accomplished. The solution was removed, plug slices were loaded directly into the wells were and the wells were sealed with molten tempered agarose. Meanwhile the gel 1% SeaKem® Gold agarose in 10x TBE was prepared and poured on into the tray. It was left to solidify for 30 minutes. Then, the gel was placed in an electrical field device, CHEF-DR III System® (Bio-Rad Laboratories, CA, USA) and the pulse time was initial switch time 6.8s, final switch time 38.4s, total runtime 19 hours; voltage 6.0 V/cm = 200V and angle 120º. The gel was stained with 0.0001% (or 1 µg/ml) ethidium bromide for 20 minutes followed by destaining in distilled water for 20 minutes.

4.3. Results

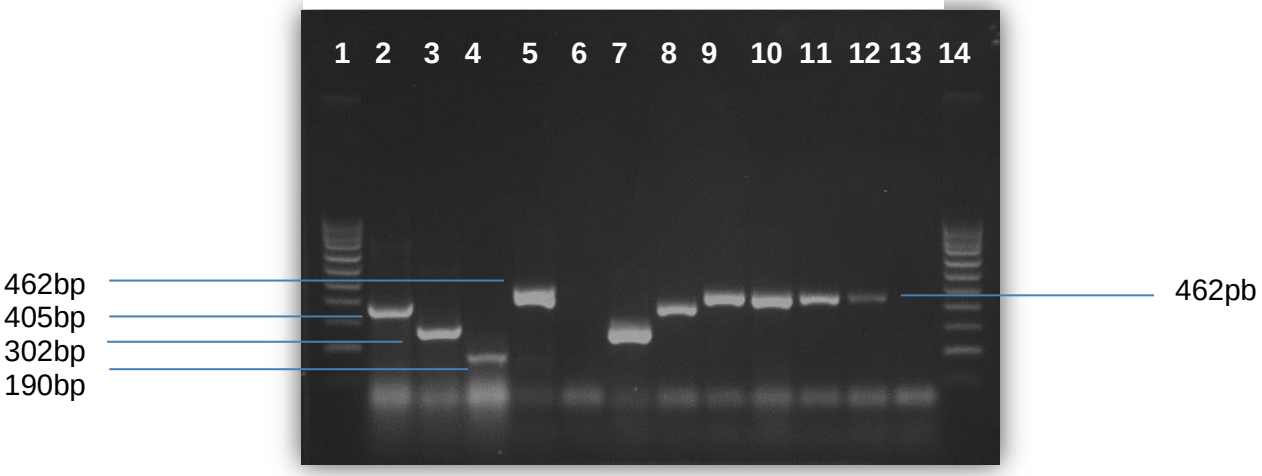
4.3.1. Detection of AmpC β-lactamase-producing

Antimicrobial resistance phenotype was confirmed for all 88 (*n*= 88) isolates by the disc diffusion method. A total of 62 (70%) isolates examined were resistant to cefoxitin. MICs of cefoxitin were performed in isolates positive to multiplex AmpC (*n*= 21). To confirm the results, VetMIC® was performed to these isolates (*n*= 21) as is illustrated in Table 9.

4.3.2. Molecular Identification of β-lactamases genes

A multiplex PCR to detect resistance gene were performed subsequently (**Erro! A origem da referência não foi encontrada.**). From the 72 Portuguese isolates analysed 18 (25%) isolates were positive to group CIT, and 3 (4%) were positive to DHA gene. A simplex multiplex was performed in order to confirm the presence of bla_{CMY-2} and bla_{DHA-1} gene. Twenty one isolates (*n*= 21; 29%) were positive for bla_{CMY-2} and bla_{DHA-1} gene. Out of 16 (*n*= 16) isolates from Denmark 11 (*n*= 11; 69%) were positive to bla_{CMY-2} gene.

Figure 30 - Multiplex PCR for ampC detection.



Lane 1 and 14 - GeneRuler DNA Ladder V 100-1000bp. From lane 2 to lane 7 - positive controls. Lane 2 – *E. coli* J53 pMG144 carrying *bla*DHA-1; lane 3 - *E. co*li J53 pMG251 carrying *bla*ACT-1;; lane 4 - *E. coli* J53 PMG 252 carrying *bla*FOX; lane 5 - *Proteus mirabilis* 1089/07 carrying *bla*CMY-2; lane 6 - *Klebsiella* Nu2936 carrying *bla*MOX; *lane 7- Klebsiella* 96D carrying *bla*MIR; lane 8 – 10/2012, lane 9 – 28/2011, lane 10 – 51/2012, lane 11- 29/2011, lane 12 – 41/2011, and lane 13 – negative control.

4.3.3. Transference of resistant genes

Plasmid transference was performed by electroporation in isolates (*n*= 32), and it was positive in 26 isolates, representing a success rate of 81% (Figure 31). In the remaining isolates transformation was not successful, meaning that there was no visible growth in selective plates (BHIA+cefotaxime 2µg/ml), despite the growth in non-selective plates.

Further, transformants were subjected to a simplex PCR to confirm the presence of *bla*$_{CMY-2}$ and *bla*$_{DHA-1}$ gene respectively.

The results demonstrated that the plasmid harbouring the *bla*$_{CMY-2}$ gene was transferred successfully to the recipient strain Genehog® *E. coli* as confirmed by the PCR (Figure 32). However when performed the simplex PCR for *bla*$_{DHA-1}$ gene all isolates were negative. Despite repeated attempts the PCR was still negative. The hypothesis suggested is that the resistance gene was harboured in the chromosome and not in a plasmid, or in a different plasmid. To confirm this hypothesis hybridization with the specific probe would confirm the chromosomal location of *bla*$_{DHA}$ gene. Due to restriction in time this was not done at the time of this study.

Figure 31 - Growth of transformants on selective plates.

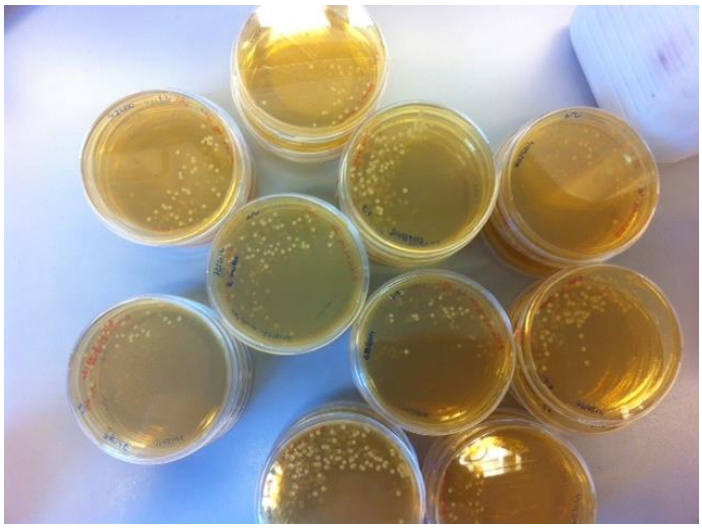
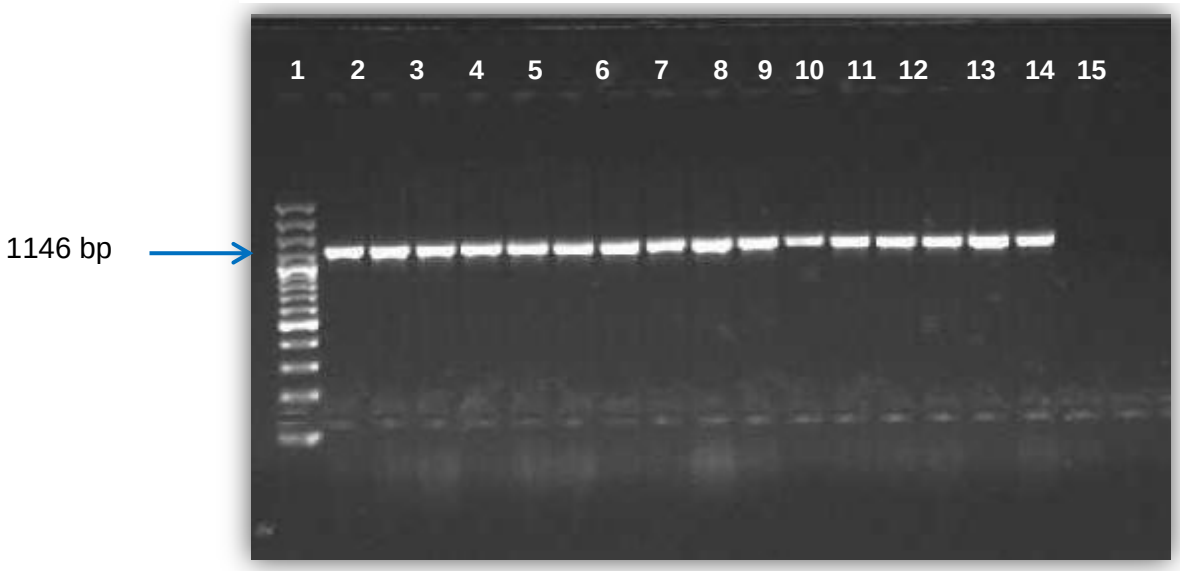


Figure 32 - Simplex PCR performed to confirm the presence of *bla*$_{CMY-2}$ gene in transformants.



Lane 1 –GeneRuler DNA Ladder Mix 100-10000bp; lane 2 - *E. coli* positive control; lane 3 - 6933/04; lane 4 - 71/2012; lane 5 - 1089/07; lane 6 - 28/2011; lane 7 - 6077/09; lane 8 - 919/09; lane 9 - 2038/05; lane10 - 4138/10; lane 11 - 6034/04; lane 12 - 25/2011; lane 13 - 41/2011; lane 14 - 434/00; lane 15 - 29/2011; lane 16 - *K. penumoniae*; lane 17 - 51/2012; lane 18 - negative control.

Table 9 – Results from resistance profile by disc diffusion, MIC and VetMIC®.

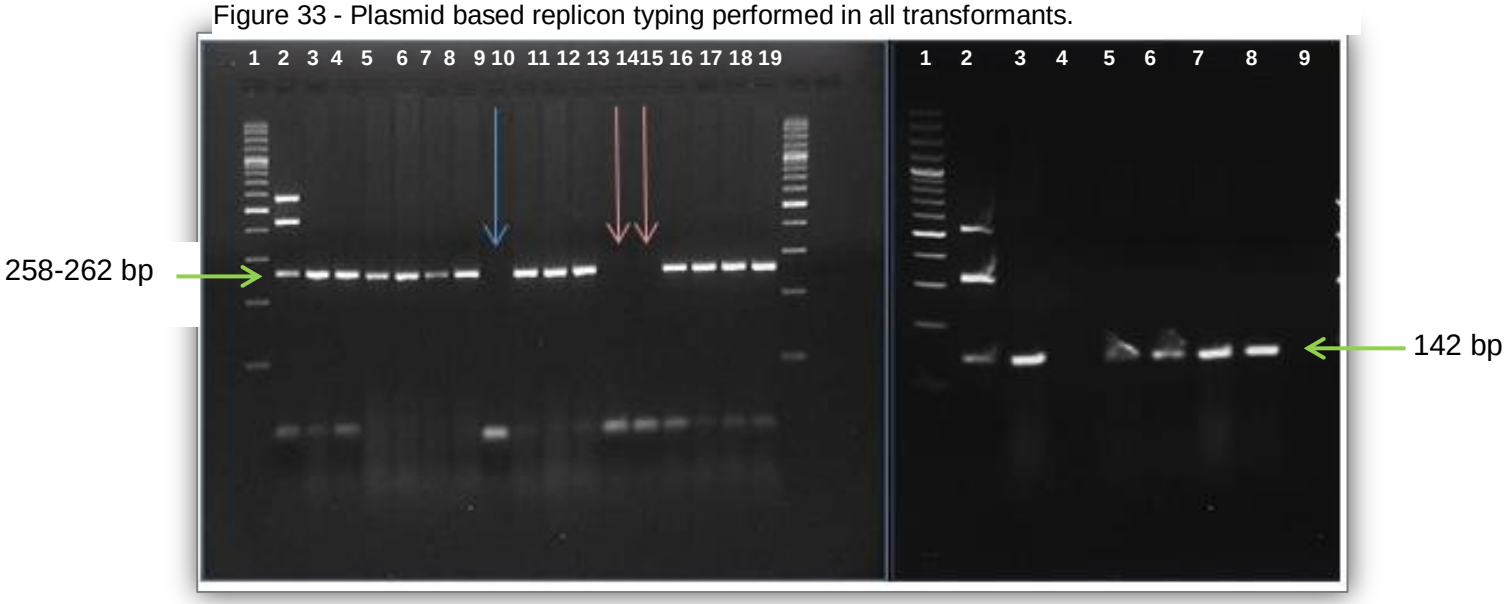
Strain No.	Susceptibility test by disc diffusion [a]								MIC	VetMIC [a]								
	AMC [b]	CTX [c]	FOX [c]	CIP [c]	CN [b]	TE [b]	SXT [b]	IPM [b]	FOX	AMP [b]	KF [b]	CTX [c]	AMC [b]	F [b]	CN [b]	SXT [b]	TE [b]	ENR [b]
6933/04	0	18	0	20	28	0	0	18	32	> 8	> 4	> 1	> 16/8	> 32	≤ 2	> 4/76	> 8	4
1089/07	0	19	12	12	0	0	0	19	32	> 8	> 4	> 1	> 16/8	> 32	> 16	> 4/76	> 8	> 4
4138/10	0	11	10	12	30	0	12	21	32	> 8	> 4	> 1	> 16/8	> 32	≤ 2	> 4/76	> 8	> 4
74/2012	0	20	9	0	0	0	0	14	16	> 8	> 4	> 1	> 16/8	> 32	16	> 4/76	> 8	4
6034/04	0	16	11	0	0	0	0	21	32	> 8	> 4	> 1	> 16/8	> 32	2	> 4/76	> 8	4
28/2011	11	9	0	0	0	0	0	27	128	> 8	> 4	> 1	> 16/8	> 32	> 16	> 4/76	> 8	> 4
K.pneumoniae	0	0	0	0	0	0	0	25	256	> 8	> 4	1	> 16/8	≤ 16	> 16	> 4/76	> 8	> 4
K.oxytoca	10	25	0	0	0	0	0	28	128	> 8	> 4	> 1	> 16/8	> 32	≤ 2	≤ 0.5/9.5	> 8	> 4
414/02	0	12	0	0	0	0	0	28	> 256	> 8	> 4	> 1	> 16/8	> 32	> 16	> 4/76	> 8	> 4
1975/05	0	11	0	0	0	0	0	28	> 256	> 8	> 4	> 1	> 16/8	> 32	> 16	> 4/76	> 8	> 4
2038/05	0	15	0	21	22	27	0	28	256	> 8	> 4	> 1	> 16/8	≤ 16	≤ 2	> 4/76	2	2
859/06	0	12	0	0	20	0	29	21	> 256	> 8	> 4	> 1	> 16/8	≤ 16	≤ 2	≤ 0,5/9,5	> 8	> 4
434/00	13	15	0	30	0	0	0	31	> 256	> 8	> 4	> 1	> 16/8	32	≤ 2	> 4/76	> 8	0,5
6346/05	9	12	0	11	10	0	23	25	> 256	> 8	> 4	> 1	> 16/8	16	> 16	0,5/9,5	> 8	> 4
4919/09	0	18	8	0	0	0	0	24	128	> 8	> 4	> 1	> 16/8	> 32	≤ 2	> 4/76	> 8	> 4
6077/09	0	11	0	0	19	0	28	22	> 256	> 8	> 4	> 1	> 16/8	≤ 16	> 16	> 4/76	> 8	> 4
25/2011	0	15	0	0	0	0	0	28	64	> 8	> 4	> 1	> 16/8	32	> 16	> 4/76	> 8	> 4
29/2011	0	15	0	0	0	0	25	24	64	> 8	> 4	> 1	> 16/8	≤ 16	> 16	> 4/76	> 8	> 4
41/2011	0	0	0	0	12	0	0	28	64	> 8	> 4	> 1	> 16/8	32	≤ 2	> 4/76	> 8	> 4
10/2012	10	0	0	0	0	0	28	23	128	> 8	> 4	> 1	> 16/8	≤ 16	> 16	> 4/76	> 8	> 4
51/2012	12	15	0	0	0	0	0	27	64	> 8	> 4	> 1	> 16/8	32	32	≤ 0,5/9,5	> 8	> 4

[a] AMC – amoxicillin-clavulanate; CTX – cefotaxime; FOX – cefoxitin; CIP – ciprofloxacin; CN – gentamicin; TE – tetracyclin; SXT – trimethoprim-sulphonamides; AMP Ampicillin; KF - cephalotin; F - nitrofurantoin; ENR – enrofloxacin. [b] Criteria according to CLSI , 2008; [c] Criteria according to CLSI, 2012

4.3.4. Plasmid Characterization

The PCR based replicon typing was performed in all transformants (*n*= 26) (Figure 33). Overall, from the Portuguese isolates (*n*= 21), seventeen (*n*= 17; 81%) were positive to IncFII incompatibility group; one isolate (*n*= 1; 5%) incorporate the IncA/C plasmid; and one isolate (*n*= 1; 5%) harboured an IncI1 plasmid. The two remaining isolates were considered non-typeable by this method. All five isolates from Denmark (*n*= 5), belonged to IncI1 replicon type (see Figure 33 and Table 10).

Figure 33 - Plasmid based replicon typing performed in all transformants.



Left image: Lanes 1 and 19- GeneRuler DNA Ladder Mix 100-10000bp; lane 2- positive controls for HIB (570bp), FIB-M (440bp) and FII (258-262bp) replicons; lane 3- 6933/04; lane 4- 1089/07; lane 5- 4138/10; lane 6- 74/2012; lane 7- 6034/04; lane 8- 28/2011; lane 9- *K.pneumoniae*; lane 10- *K.oxytoca*; lane 11- 414/02; lane 12- 2038/05; lane 13- 859/06; lane 14- 6346/05; lane 15- 4919/09; lane 16- 10/2012; lane 17- 51/2012. The blue narrow refers to a non-typeable plasmid and the pink narrow to the IncI1 and IncA/C replicon type. Right image: Lane 1 GeneRuler DNA Ladder Mix 100-10000bp; lane 2- positive controls for HI1 (534bp), HI2 (327bp) and I1 (142bp) replicons; lane 3- 30029; lane 5- 29969; lane 6- 28463; lane 7- 28464; lane 8- 30104; lane 9- 29870 (negative strain).

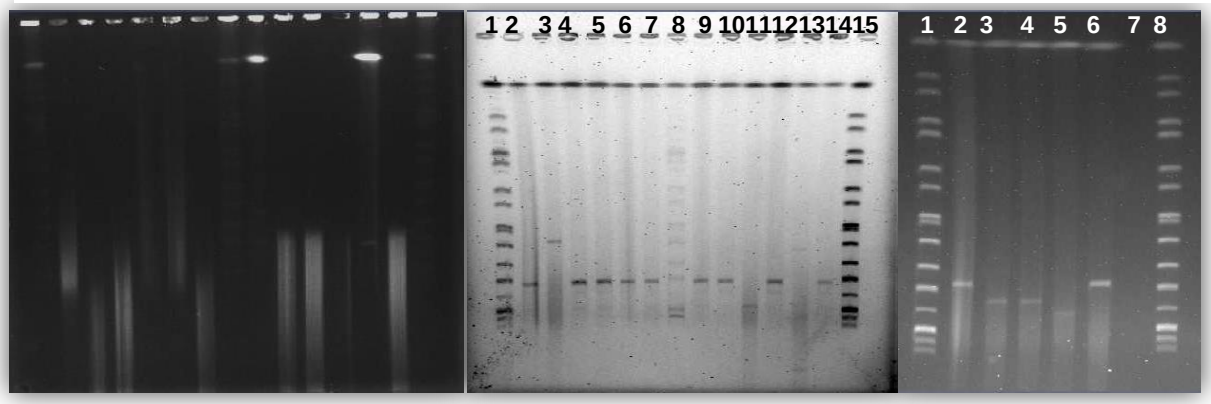
Table 10 - PCR based-replicon typing results.

Origin isolates	bla_{CMY-2} gene				bla_{DHA-1} gene	Total
	repFII	repIncI1	repA/C	non-typeable	repFII	
Animal isolates (*n*=23)	13	5	1	1	3	23
Human isolates (*n*=3)	1	1	-	1	-	3
Total (*n*=26)	14 (54%)	6 (23%)	1 (4%)	2 (8%)	3 (11%)	26

4.3.5. S1 nuclease PFGE

S1-PFGE was performed in all transformants (*n*= 26). As it is shown in Figure 34, left image there is DNA degradation (smear pattern) in all isolates. To overcome this situation thiourea (100µM) was added to the tris-containing buffer (Zhang *et al*., 2004). In this study the use of thiourea solved the problem of DNA degradation. Plasmids from Portuguese isolates belonged to three different incompatibility groups: IncFII/F of ca. 78 Kb, one isolate IncA/C of ca. 140 Kb; and one IncI1 of ca. 86 Kb. Two isolates: *Klebsiella pneumoniae* and *E. coli* (human origin) were non-typeable by PBRT method, but in S1-PFGE gel plasmids were detected. The plasmids from Danish isolates were all IncI1 ranging from 55 to 104 Kb sized.

Figure 34 - S1 nuclease PFGE performed in transformants.



Middle image: Lanes 1, 8 and 15 - *Salmonella enterica* serovar Branderup molecular mass marker; lane 2- 6933/04; lane 3- 6346/05; lane 4- 74/2012, lane 5- 1089/07, lane 6- 4919/09, lane 7- 4138/10; lane 9- 6034/04, lane 10- *E. coli*; lane 11- *K. pneumoniae*; lane 12- 28/2011; lane 13- 1975/05; lane 14- 51/2012. Right image: Lanes 1 and 8 - *Salmonella enterica* serovar Branderup molecular mass marker; lane 2- 30104; lane 3- 28463; lane 4- 28464; lane 5- 29969; lane 6 - 30029.

4.3.6. Plasmid analysis

I1 and FII plasmids were submitted to pMLST as described in the pubmed database. All isolates that belonged to IncFII (*n*= 17) incompatibility group were sequenced and the results according to the FAB formula were: F2:A-:B-. Amplicons were compared with known allelic variants and sequence types (ST) assigned using the pMLST database (http://pubmlst.org/plasmid) (García-Fernández *et al*., 2008). Out of 6 (*n*= 6) Inc1 plasmids, 3 (*n*= 3) were classified as ST2 and the remaining three were considered non-typeable by pMLST (Table 11). In two out of the three non-typeable a new allele *ardA* 20 was reported in this study for the locus ardA. Antimicrobial susceptibility testing on transformants was done to detect co-transference of antimicrobial resistance. In IncFII plasmids the results showed additional resistance to other antimicrobials agents such as ciprofloxacin, gentamicin, tetracycline's and sulphametoxazole-trimethoprim; whereas the IncI1 plasmids did not transfer any additional resistance besides to β-lactam antimicrobials.

Table 11 - Plasmid characterization.

		Plasmid characterization				
Strain	Origin	*bla* gene (s)	Replicon typing	Plasmid size(Kb)	pMLST	Co-transferred resistance[c]
PT6933/04	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT1089/07	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT4138/10	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT74/2012	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT6034/04	Cat	CMY-2	FII	78.2	F2:A-:B-	AMC, FOX, CIP, CN, TE, SXT
PT28/2011	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT*K. pneumoniae*	Dog	CMY-2	Negative	33.3	-	AMC, CTX, FOX
PT*K.oxytoca*	Dog	DHA-1	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT414/02	Dog	DHA-1	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT1975/05	Human	CMY-2	Negative	28,8 ; 104-138[a]	-	AMC, CTX, FOX
PT2038/05	Human	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT859/06	Human	CMY-2	I1	86	ST2	AMC, CTX, FOX
PT434/00	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT6346/05	Cat	CMY-2	A/C	138.9	F2:A-:B-	AMC, CTX, FOX
PT4919/09	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, FOX, CIP, CN, TE, SXT
PT6077/09	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT25/2011	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT29/2011	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT41/2011	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT10/2012	Dog	DHA-1	FII	78.2	F2:A-:B-	AMC, CTX, FOX, CIP, CN, TE, SXT
PT51/2012	Dog	CMY-2	FII	78.2	F2:A-:B-	AMC FOX, CIP, CN, TE, SXT
DK28464	Dog	CMY-2	I1	54.7-78.2[b]	NT[d]	AMC, CTX, FOX
DK28463	Dog	CMY-2	I1	54.7-78.2[b]	NT[d]	AMC, CTX, FOX
DK30029	Dog	CMY-2	I1	78.2-104[b]	ST2	AMC, CTX, FOX
DK29969	Dog	CMY-2	I1	54.7	NT[d]	AMC, CTX, FOX
DK30104	Dog	CMY-2	I1	78.2-104[b]	ST2	AMC, CTX, FOX

[a] Two plasmids were detected;
[b] plasmid size between these two values;
[c] AMC - amoxicillin-clavulanate; CTX - cefotaxime; FOX - cefoxitin; CIP - ciprofloxacin; CN - gentamicin; TE - tetracycline; SXT - trimethoprim-sulphonamides;
[d] NT – non-typeable

4.4. Discussion

The objective of this study was to assess the presence of pAmpC genes among Enterobactereaceae isolates collected from companion animals and human clinical isolates and to subsequently characterize positive isolates by using molecular methods for plasmid characterization.

From the 49 *E. coli* isolates the pAmpC genes were detected in 33 *E. coli* isolates, while in *Klebsiella* spp isolates was detected in 3 of the 9 isolates. In *Proteus* spp the pAmpC gene was detected in 5 of 10 isolates and in 1 out of 18 *Enterobacter* spp. isolates. *E. coli* produces chromosomal AmpC β-lactamases at low level and may hyper produce due to mutations in promoter or attenuator region (Siu, Lu, Chen, Lin & Chang, 2003). *E. coli* isolates which were resistant to cefoxitin but did not carry any gene belonging to the CIT group analysed by PCR multiplex, may either hyperproduce cAmpCs or produce a novel pAmpC which is not detected by the multiplex PCR (Singtohin *et al*., 2010) Both *K. pneumonia* and *Proteus* spp. lacks the cAmpCs (Siu *et al*., 2003).

In our study a dominance of CMY-2 type plasmid-mediated AmpC (26%) was detected in isolates from animal and human origin consistent with worldwide observations (Naseer, Haldorsen, Simonsen & Sundsfjord, 2010; Fam *et al*., 2013;). In a recent study, bla_{CMY-2} gene was detected in one third of the 150 canine and feline *E. coli* isolates from urinary tract infection, suggesting that was the most common β-lactamase (O'Keefe, Hutton, Schifferli & Rankin, 2010). According to Carattoli (2005) the increase of Enterobacteriaceae producing cephalosporinase detected in dogs and cats is due to the diffuse off-label veterinary use of extended spectrum cephalosporins (Carattoli *et al*., 2005b). However in a study conducted by Zhao (2001) revealed that many isolates possessing bla_{CMY-2} genes were recovered from animals that had no history of exposure to cephamycins and extended-spectrum cephalosporins (Zhao *et al*., 2001). Reports refer that AmpC enzyme is less common than ESBL among Enterobacteriaceae. However, in a study conducted in a Canadian hospital the rates of plasmid AmpC β-lactamases were higher than the rate for extended-spectrum β-lactamases (Mataseje *et al*., 2010).

In addition we also observed a low prevalence of *bla*$_{DHA-1}$ (3%) gene, and these results are in agreement with other studies where this gene was detected in only two isolates from dogs in the Republic of Korea (Tamang *et al*., 2012). The occurrence of *bla*$_{DHA-1}$ has previously been described in human clinical *E. coli* isolates, and is the most prevalent gene in china (Li *et al*., 2008).

In the present study the majority of our isolates, *bla*$_{CMY-2}$ gene was able to be transferred successfully by transformation to a recipient *E. coli* Genehog® strain. The *bla*$_{DHA-1}$ gene interestingly did not transfer to recipient strains, despite multiple attempts which indicate that this genetic determinant could be located in the chromosome and not in a plasmid. To assess this hypothesis southern hybridization could be determined to confirm, however it was out of the scope of this study, and time was a limiting factor.

Both *bla*$_{CMY-2}$ and *bla*$_{DHA-1}$ genes were plasmid encoded. *bla*$_{CMY-2}$ (*n*= 23) gene belonged to replicon type IncF (*n*= 14; 54%), followed by IncI1 (*n*= 6; 23%) replicon type and one isolate (n= 1; 4%) harboured an A/C plasmid. Two isolates (*n*= 2; 8%) were considered "non-typeable" by this method. FII plasmid sized 78.2 Kb, IncI1 sized from 54 to 104 Kb, while the A/C plasmid sized 138.9 Kb. Our results showed high prevalence of IncFII plasmids (54%) and these results are consistent with previous studies since IncFII plasmids are narrow host range representing one of the most common plasmid types in clinical relevant Enterobacteriaceae (Carattoli, 2009). IncFII plasmids are known by encoding important resistant genes, as exemplified by the spread of *bla*$_{CTX-M-15}$ genes (Boyd *et al*., 2004). Recently *bla*$_{NDM-1}$ gene was reported to be encoded in an IncFII plasmid. This plasmid type spreads efficiently among bacteria which is of great concern (Bonnin, Poirel, Carattoli & Nordmann, 2012). For example in a report this replicon type was reported in *E. coli* from faeces of healthy with no history of antimicrobial use, and also in the faecal flora from healthy birds in the USA (Johnson *et al*., 2007). Overall, IncFII plasmids showed highest occurrence among typed resistant plasmid in human and animal isolates detected in most countries (Sherley, Gordon & Collignon, 2003; Johnson *et al*., 2007). It is possible to assume that the detection of the same gene (*bla*$_{CMY-2}$) within the same plasmid (IncFII) is responsible for the spread and diffusion of this plasmid. In our results, the *bla*$_{DHA-1}$ (*n*= 3; 11%) gene was encoded in a 78.2 Kb IncFII plasmid. This findings is in agreement with previous reports (Carattoli, 2009).

I1 incompatibility group was detected in 6 (*n*= 6; 23%) isolates: one (*n*= 1) *E. coli* isolate from human origin, and in all 5 (*n*= 5) isolates from Denmark. In other studies, this plasmid type is frequently reported as plasmid encoding (Carattoli, 2009; Mataseje *et al*., 2010; Naseer *et*

al., 2010; Dierikx *et al*., 2012). IncI1 plasmids carrying the *bla*$_{CMY-2}$ gene was reported in canine *E. coli* isolates from Italy (Winokur, Vonstein, Hoffman & Uhlenhopp, 2001). In another study conducted in Denmark IncI1 plasmids were the most common replicon type among dogs (Damborg, Gaustad, Olsen & Guardabassi, 2011). IncI1 plasmid carrying CMY-2 gene was reported in Norway, as the most common replicon type among humans (Naseer *et al*., 2010). The host range and the reporting in several countries indicates high mobility (Winokur *et al*., 2001). Comparing with our results, the I1 replicon typing was reported in an *E. coli* isolate from human origin, and in this work only three human isolates were considered, which represents a limiting factor.

One (*n*= 1) *bla*$_{CMY-2}$ gene was located in an A/C plasmid, and it is consistent with other authors. In a study conducted by Johnson (2007) on *E. coli* isolates from faecal flora of healthy animals demonstrated that the repA/C replicon tying was absent, and when compared with the results obtained in healthy humans the prevalence was 1% (Johnson *et al*., 2007). However, in the United States of America the majority of *bla*$_{CMY-2}$ gene was carried on a IncA/C plasmid (Welch *et al*., 2007). The prevalence of repA/C plasmids is low but when antimicrobial selective pressure is active it can became more prevalent (Winokur *et al*., 2001). These large plasmids have been detected in Enterobacteriaceae family and in other gram-negative bacteria (Call *et al*., 2010), from human and animal origin isolates, such as beef, chicken (Winokur *et al*., 2001).

Overall, PBRT demonstrated that is a good method for detecting replicon types in plasmids allows tracing their diffusion and detecting the mobilization capacity of a different gene among plasmids (Hopkins *et al*., 2006). However this scheme still has some limitations, since the classification is currently based on plasmids belonging to the 18 of the 26 known Inc groups and novel replicons cannot be identified (Carattoli, 2009; Lindsey, Fedorka-Cray, Frye & Meinersmann, 2009). This fact may explain why plasmids from one *E. coli* isolate and one *Klebsiella pneumoniae* in our study were considered “non-typeable”. The most precise method to analyse plasmids is based on the determination of the full-length DNA sequence, and so far more than 800 plasmids from *Gammaproteobacteria* have been fully sequenced (http://www.ncbi.nlm.nih.gov/genome/), contributing to the identification of novel plasmid families (Carattoli, 2009).

Since these plasmids are very common in Enterobacteriaceae a further typing scheme has been proposed, using pMLST (García-Fernández *et al*., 2008). IncI1 plasmids carrying AmpC have been described recently in *E. coli* and *Salmonella* (Hopkins *et al*., 2006). The presence *pili* type IV in these plasmids contributes to adhesion of membranes and invasion

of bacterial pathogens which represents a crucial factor for the positive selection of these plasmids (Kim & Komano, 1997). Briefly, the pMLST method involved PCR amplifying and sequencing genes essential for replication and maintenance, which include: *pill*, a gene within the pil locus involved in pilus biogenesis; *sogS*, a primase involved in discontinuous plasmid DNA replication; *ardA*, encodes a type-I restriction enzyme, *repI1*, the RNAI antisense regulating system in the IncI1 replication system and the intragenic *trbA*- pndC region involved in maintenance and plasmid transfer respectively (García-Fernández *et al*., 2008). The IncF family contains a varied group of plasmids, whose relatedness and nomenclature are often complex. One peculiar feature is that IncF plasmids are limited by host to the family Enterobacteriaceae and rely on both self-encoded and host-encoded factors for duplication. IncF plasmids need DNA gyrase, DnaB, DnaC, DnaG, single strand binding and DNA polymerase III proteins for their replication. Since the IncF plasmids are considered to be heterogeneous in size and carry multiple replicons, pMLST allows the characterization into homogenous groups according to their phylogenetic relatedness (Villa, García-Fernández, Fortini & Carattoli, 2010). IncF plasmid contains the FII replicon which is regulated by CopA, a constitutively synthesized 90 antisense-RNA, which is usually silent. The copA region of the FII replicon is involved in the control of replication and incompatibility beghvaiour. FIA and FIB replicons are regulated by iterons, in cis-negative binding sites of the replication protein RepA and RepB respectively (Villa, García-Fernández, Fortini & Carattoli, 2010). Plasmid multilocus sequence typing (pMLST) in our study was performed to characterize IncFII, IncI1, plasmids, however it can be used also to characterize also IncN and IncHI2 plasmids (García-Fernández *et al*., 2008).

Within replicon type I1 using the pMLST (http://pubmlst.org/plasmid/) we determined that these plasmids belonged to sequence type ST-2 (*n*= 3; 50%), and the remaining 3 (*n*= 3; 50%) were considered non-typeable. These results have been previously shown in *E. coli* repI1 plasmids harbouring bla_{CMY-2} isolated from dogs in Italy (García-Fernández *et al*., 2008). RepFII were characterized as F2;FIA-;FIB- according to the formula (FII; FIA; FIB).

The resistance among Enterobacteriaceae to β-lactams is increasing both in humans and animals and there is no specific β- lactamase that is restricted only to animals or humans because most of them have been reported in both (Trott, 2012). This fact is interesting and not very surprising since we share the same environment and the β-lactams are widely used whether in veterinary medicine whether in human facilities (Pitout, Nordmann, Laupland & Poirel, 2005). Overall, the results obtained by the PBRT indicates a high prevalence of some replicon types, such as repF, repI and repA/C associated with important resistance genes, bla_{CMY-2}. Besides direct transfer of resistance, resistance mechanism may possibly be

acquired indirectly, through the transfer of resistance genes from bacteria of animal origin to humans. Some studies have pointed out that the possibility of this type of transfer. Winokur (2001) refers to the possibility of CMY-2 producing *E. coli* transference between animals and humans due to the association with ISEcp1 (Winokur *et al*., 2001; Naseer *et al*., 2010). Large plasmids, carrying resistance genes and transfer-associated genes, may be potential factors for dissemination of antibiotic resistance (Huang *et al*., 2012).

5. Conclusion and further studies

The results of the study regarding antimicrobial use indicate that widespread of broad spectrum antimicrobials was observed in both dogs and cats, as antimicrobials deemed as critically important in human medicine. Overall, antimicrobial use in companion animals was according to guidelines implemented in other countries and to previous studies performed. However, part of the antimicrobial use may be inadequately justified, and there is a need for further surveys about indication-based use of antimicrobials in veterinary medicine. The introduction of antimicrobial use guidelines has been shown to lead to more appropriate use of antimicrobials, because it could reduce prescribing errors leading to more prudent antimicrobial use, and is therefore recommended.

This study has also revealed the occurrence of plasmid mediated AmpC β-lactamase producing strains in clinically important bacterial isolates. Clinical laboratories should test FOX-resistant isolates for the presence of pAmpC enzymes, in order to improve detection of this emerging resistance phenotype. Since AmpC β-lactamase production is frequently associated with multidrug resistance, the dissemination of this AmpC β-lactamase encoding plasmids may facilitate the spread of resistance genes, such as bla_{CMY-2} among different members of Enterobacteriaceae. To analyse the potential zoonotic transmission of pAmpC to humans, further research would be necessary to confirm this association.

In summary, this work highlights the importance of monitoring antimicrobial usage, in order to reduce the selective pressure, which can lead to development of antimicrobial resistance.

6. References

N.B.: This section also includes the references supporting the complementary annexes.

Aarestrup, F. M. & Wegener, H. C. (1999). The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in Campylobacter and Escherichia coli. *Microbes and Infection*, *1*(8), 639–44. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10611741

Abbott, S. (2008). Enterobacter, Citrobacter, Serratia, Plesiomonas, and other Enterobacteriaceae. In Murray, P. R., Baron, E.J., Jorgensen, J.H., Landry, M.L., Pfaller, M. (Eds.), *Manual of Clinical Microbiology* (Vol. 1, pp. 699–710). Washington DC: ASM Press.

Abraham, E. P. & Newton, G. G. (1961) The structure of cephalosprin. *Biochemical Journal*, *79*(2), 377-393. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1205850

Alvarez, M., Tran, J. H., Chow, N. & Jacoby, G. A. (2004). Epidemiology of Conjugative Plasmid-Mediated AmpC β-Lactamases in the United States. *Antimicrobial Agents and Chemotherapy*, *48*(2), 533–537. doi:10.1128/AAC.48.2.533

Ambler, R. P. (1980). The Structure of beta-Lactamases. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *289*(1036), 321–331. doi:10.1098/rstb.1980.0049

American Veterinary Medical Association (AVMA). (2006). *American Association of Feline Practitioners/American Animal Hospital Association Basic Guidelines of Judicious Therapeutic Use of Antimicrobials.* Retrieved August 30, 2013, from http://www.avma.org/issues/policy/jtua_aafp_aaha.asp

American Veterinary Medical Association (AVMA). (2007). *Position statement: Judicious Use of Antimicrobials.* Retrieved August 20, from www. avma.org

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology*, *1* (December), 134. doi:10.3389/fmicb.2010.00134

Anderson, A. D., Nelson, J. M., Rossiter, S. & Angulo, F. J. (2003). Public health consequences of use of antimicrobial agents in food animals in the United States. *Microbial Drug Resistance*, *9*(4), 373–9. doi:10.1089/107662903322762815

Arlet, G., Barrett, T. J., Butaye, P., Cloeckaert, A., Mulvey, M. R. & White, D. G. (2006). Salmonella resistant to extended-spectrum cephalosporins: prevalence and epidemiology. *Microbes and Infection*, *8*(7), 1945–54. doi:10.1016/j.micinf.2005.12.029

Associação Portuguesa da Indústria Farmacêutica (Apifarma). (2011). Simposium Veterrinário. Retrieved July 23, from ww.apifarma.pt/simposiumvet/Paginas/default.aspx

Babic, M., Hujer, A. M. & Bonomo, R. A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *9*(3), 142–56. doi:10.1016/j.drup.2006.05.005

Baldo, B. A. (1999). Penicillins and cephalosporins as allergens--structural aspects of recognition and cross-reactions. *Journal of the British Society for Allergy and Clinical Immunology*, *29*(6), 744–9. doi:10.1046/j.1365-2222.1999.00575.x

Bambeke, F.V., Barcia-Macay, Maritza., Lemaire, S. & Tulkens, P.M. (2006). Cellular Pharmacodynamics and Pharmacokinetics of Antibiotics: Current Views and Perspectives. *Current Opinion in Drug Discovery & Development*, *9(2)*, 218-230. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16566292

Barlow, M. & Hall, B. G. (2002). Origin and Evolution of the AmpC - β-Lactamases of Citrobacter freundii. *Antimicrobial Agents and Chemotherapy*, *46*(5), 1190–1198. doi:10.1128/AAC.46.5.1190-1198.2002

Barthélémy, M., Peduzzi, J. & Labia, R. (1985). Distinction between the primary structures of TEM-1 and TEM-2 beta-lactamases. *Annales de l´Institut Pasteur*, *136A*, 311. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3901878

Barza, M. & Travers, K. (2002). Excess Infections Due to Antimicrobial Resistance: The " Attributable Fraction". *Clinical Infectious Diseases*, *34*(Suppl 3), S126–130. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11988883

Bauernfeind, A., Schneider, I., Jungwirth, R., Sahly, H. & Ullmann, U. (1999). A novel type of AmpC beta-lactamase, ACC-1, produced by a Klebsiella pneumoniae strain causing nosocomial pneumonia. *Antimicrobial Agents and Chemotherapy*, *43*(8), 1924–31. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC89392/

Bebrone, C. (2007). Metallo-beta-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. *Biochemical Pharmacology*, *74*(12), 1686–701. doi:10.1016/j.bcp.2007.05.021

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, *153 Suppl*(January), S347–57. doi:10.1038/sj.bjp.0707607

Bergström, S. & Normark, S. (1979). β-lactam resistance in clinical isolates of Escherichia coli caused by elevated production of the ampC-mediated chromosomal β-lactamase. *Antimicrobial Agents and Chemotherapy*, *16*(4), 427–33. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC352877/

Beveridge, T. J. & Graham, L. L. (1991). Surface Layers of Bacteria. *Microbiological Reviews*, *55*(4), 684–705. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC372843/

Beveridge, T. J. (1999). Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *Journal of Bacteriology*, *181*(16), 4725–4733. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC93954/

Bimboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, *7*(6), 1513–1523. doi:10.1093/nar/7.6.1513

Bonnin, R. A., Poirel, L., Carattoli, A. & Nordmann, P. (2012). Characterization of an IncFII Plasmid Encoding NDM-1 from Escherichia coli ST131. *PloS one*, *7*(4). doi:10.1371/journal.pone.0034752.s001

Botana, L.M., Landoni, F., Martín-Jiménez, T. (2002). *Farmacología y Terapéutica Veterinaria*. Madrid: McGraw – Hill Interamericana.

Boultwood, J. (1994). P*rotocols for Gene Analysis: Physical Mapping of the Human Genome by Pulsed Field Gel Electrophoresis.* Oxford: Humana Press

Boyd, D. A., Tyler, S., Christianson, S., Muller, M. P., Willey, B. M., Gardam, M., Brice, E.,Nordman, P. & Mulvey, M.R. (2004). Complete Nucleotide Sequence of a 92-Kilobase Plasmid Harboring the Beta-Lactamase Involved in an Outbreak in Long-Term-Care Facilities in Toronto, Canada. *Antimicrobial Agents and Chemotherapy*, *48*(10), 3758–3764. doi:10.1128/AAC.48.10.3758

Bradford, P. A. (2001). Extended-Spectrum β-Lactamases in the 21st Century: Characterization , Epidemiology , and Detection of This Important Resistance Threat. *Clinical Microbiology Reviews*, *14*(4), 933–951. doi:10.1128/CMR.14.4.933

Brisse, S., Grimont, F. & Grimont, P. A. D. (2006). The Genus Klebsiella. In M. Dworkin & E. Falkow, S.; Rosenberg, E.; Schleifer, K.-H.; Stackebrandt (Eds.), *The Prokaryotes* (Third edit., pp. 159–196). Springer New York.

British Small Animal Veterinary Association (BSAVA). (2013). *BSAVA Guide to the Use of Veterinary Medicines*. Retrieved August 30, 2013, from http://www.bsava.com/

Bush, K. (1988). β-lactamase Inhibitors from Laboratory to Clinic. *Clinical Microbiology Reviews*, *1*(1), 109–23. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC358033/

Bush, K. (2010). Bench-to-bedside review: The role of beta-lactamases in antibiotic-resistant Gram-negative infections. *Critical Care (London, England)*, *14*(3), 224. doi:10.1186/cc8892

Bush, K., Jacoby, G. A. & Medeiros, A. A. (1995). A Functional Classification Scheme for β-Lactamases and Its Correlation with Molecular Structure. *Antimicrobial Agents and Chemotherapy*, *39*(6), 1211–1233. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC162717/pdf/391211.pdf

Bush, K. & Jacoby, G. A. (2010). Updated Functional Classification of β-lactamases. *Antimicrobial Agents and Chemotherapy*, *54*(3), 969–76. doi:10.1128/AAC.01009-09

Buynak, J. D. (2006). Understanding the longevity of the β-lactam antibiotics and of antibiotic/beta-lactamase inhibitor combinations. *Biochemical Pharmacology*, *71*(7), 930–40. doi:10.1016/j.bcp.2005.11.012

Call, D. R., Singer, R. S., Meng, D., Broschat, S. L., Orfe, L. H., Anderson, J. M., Herndon, D.R.,Kappmeyer, L.S.,Daniels, J.B. & Besser, T. E. (2010). blaCMY-2-positive IncA/C plasmids from Escherichia coli and Salmonella enterica are a distinct component of a larger lineage of plasmids. *Antimicrobial Agents and Chemotherapy*, *54*(2), 590–6. doi:10.1128/AAC.00055-09

Canadian Veterinary Medical Association (CVMA). (2000). Guidelines on the prudent use of antimicrobial drugs in animals. 2000. Retrieved August 20, 2013, from http://canadianveterinarians.net

Canadian Veterinary Medical Association (CVMA). (2010). Extra-label Drug Use (ELDU)-position statement. 2010. Retrieved August 15, from http://canadianveterinarians.net

Cantón, R. & Coque, T. M. (2006). The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology*, *9*(5), 466–75. doi:10.1016/j.mib.2006.08.011

Carattoli, A. (2003). Plasmid-mediated antimicrobial resistance in Salmonella enterica. *Current Issues in Molecular Biology*, *5*(4), 113–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12921226

Carattoli, A. (2009). Resistance Plasmid Families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy*, *53*(6), 2227–38. doi:10.1128/AAC.01707-08

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L. & Threlfall, E. J. (2005a). Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods*, *63*(3), 219–28. doi:10.1016/j.mimet.2005.03.018

Carattoli, A., García-Fernández, A., Varesi, P., Fortini, D., Gerardi, S., Penni, A.,Mancini, C. & Giordano, A. (2008). Molecular Epidemiology of Escherichia coli producing extended-spectrum β-lactamases isolated in Rome, Italy. *Journal of Clinical Microbiology*, *46*(1), 103–8. doi:10.1128/JCM.01542-07

Carattoli, A., Lovari, S., Franco, A., Matteo, P. Di., Battisti, A. & Cordaro, G. (2005b). Extended-Spectrum β -Lactamases in Escherichia coli Isolated from Dogs and Cats in Rome , Italy , from 2001 to 2003. *Antimicrobial Agents and Chemotherapy*, *49*(2), 2001–2004. doi:10.1128/AAC.49.2.833

Chaibi, E. B., Sirot, D., Paul, G. & Labia, R. (1999). Inhibitor-resistant TEM -lactamases: phenotypic, genetic and biochemical characteristics. *Journal of Antimicrobial Chemotherapy*, *43*(4), 447–458. doi:10.1093/jac/43.4.447

Chambers, H.F. (2010). Penicillins and β-Lactam Inhibitors. In Mandel, G.L., Bennett, J.E., Dolin, R. (Eds). *Principles and Practice of Infectious Diseases*. (pp. 309-321). London: Elsevier, Churchill, Livingstone

Chauvin, C., Beloeil, P. A., Orand, J.P., Sanders, P. & Madec, F. (2002). A survey of group-level antibiotic prescriptions in pig production in France. *Preventive Veterinary Medicine*, *55*(2), 109–20. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12350315

Choe, C. H., Bouhaouala, S. S., Brook, I., Elliott, B. & Knudson, G. B. (2000). In Vitro Development of Resistance to Ofloxacin and Doxycycline in Bacillus anthracis Sterne. *Antimicrobial Agents and Chemotherapy*, 3–4. doi:10.1128/AAC.44.6.1766-1766.2000.Updated

Clinical Laboratory Standards Institute (CLSI). (2008).. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. Approved Standard: Third Edition M31-A3. CLSI, Wayne, PA, USA.

Clinical and Laboratory Standards Institute (CLSI). (2012). Performance Standards for Antimicrobial Susceptibility Testing M100-S22. CLSI, Wayne, PA, USA.

Coates, A. R. & Halls, G. Hu, Y. (2011). Novel classes of antibiotics or more the same? The British Journal of Pharmacology, 163(1),184-194. doi:10.1111/j.1476-5381.2011.01250

Coenen, S., Ferech, M., Dvorakova, K., Hendrickx, E., Suetens, C. & Goossens, H. (2006). European Surveillance of Antimicrobial Consumption (ESAC): outpatient cephalosporin use in Europe. *Journal of Antimicrobial Chemotherapy*, *58*(2), 413–7. doi:10.1093/jac/dkl185

Coudron, P. E., Hanson, N. D. & Climo, M. W. (2003). Occurrence of Extended-Spectrum and AmpC Beta-Lactamases in Bloodstream Isolates of Klebsiella pneumoniae: Isolates Harbor Plasmid-Mediated FOX-5 and ACT-1 AmpC Beta-Lactamases. *Journal of Clinical Microbiology*, *41*(2), 772–777. doi:10.1128/JCM.41.2.772

Damborg, P., Gaustad, I.B., Olsen, J.E., Guardabassi, L. (2011). Selection of CMY-2 producing Escherichia coli in the faecal flora of dogs treated with cephalexin. *Veterinary Microbiology* (2010). doi:10.1016/j.vetmic.2011.03.015

Danish Small Animal Veterinary Association (SvHKS). (2013). Antibiotic Use Guidelines for Companion Animal Pratice. Denmark. ISBN 978-87-870703-0-0

DANMAP 2012. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032

Davies, J. & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology Mollecular Biology Reviews*, *74*(3), 417-33. doi: 10.1128/MMBR.00016-10

Decreto-Lei n.º 148/2008 de 29 de Julho. Diário da República nº145 – I Série. Minnistério da Agicultura, do Desenvolvimento Rural e das Pescas. Lisboa. Retrieved from: http://dre.pt/pdf1sdip/2008/07/14500/0504805095.PDF

Direcção Geral de Alimentação e Veterinária (DGAV). (2011). *Relatório nacional de monitorização de consumo de antimicrobianos* – Portugal. Lisboa: Direcção-Geral de Alimentação e Veterinária & Direcção de Serviços de Medicamentos e Produtos de Uso Veterinário. Retrieved August 28, from http://www.dgv.min-agricultura.pt/

Dierikx, C. M., van Duijkeren, E., Schoormans, A. H. W., van Essen-Zandbergen, A., Veldman, K., Kant, A., Huijsdens, X.W., van der Zwaluw, K., Wagenaar, J.A. & Mevius, D. J. (2012). Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *Journal of Antimicrobial Chemotherapy*, *67*(6), 1368–74. doi:10.1093/jac/dks049

Donnenberg, M. (2010). Enterobacteriaceae. In Mandel, G.L., Bennett, J.E., Dolin, R. (Eds). *Principles and Practice of Infectious Diseases* (pp. 2567–2586). London: Elsevier, Churchill, Livingstone

Donowitz, G. R. & Mandell, G. L. (1988). Drug therapy. Beta-lactam antibiotics (2). *The New England Journal of Medicine*, *318*(8), 490–500. doi:10.1056/NEJM198802253180806

Drawz, S. M. & Bonomo, R. A. (2010). Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews*, *23*(1), 160–201. doi:10.1128/CMR.00037-09

Du Bois, S. K., Marriott, M. S. & Amyes, S. G. (1995). TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection , structure and function. *Journal of Antimicrobial Chemotherapy*, *2*, 7–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7768784

Edelstein, M., Pimkin, M., Palagin, I., Edelstein, I. & Stratchounski, L. (2003). Prevalence and Molecular Epidemiology of CTX-M Extended-Spectrum β-Lactamase-Producing Escherichia coli and Klebsiella pneumoniae in Russian Hospitals. *Antimicrobial Agents and Chemotherapy*, *47*(12), 3724–3732. doi:10.1128/AAC.47.12.3724

Elseviers, M. M., Ferech, M., Vander Stichele, R. H. & Goossens, H. (2007). Antibiotic use in ambulatory care in Europe (ESAC data 1997-2002): trends, regional differences and seasonal fluctuations. *Pharmacoepidemiology and Drug Safety*, *16*(1), 115–23. doi:10.1002/pds.1244

European Comission. (2005). Ban on antibiotics as growth promoters in animal feed enters into effect. Retrieved July 20 from http://europa.eu/rapid/press-release_IP-05-1687_en.htm?locale=en

European Committee on Antimicrobial Susceptibility testing (EUCAST). (2013). EUCAST definitions of clinical breakpoints and epidemiological cut-off values. Retrieved July 12, from http://eucast.org/

European Medecines Agency (EMA). (2009a). The bacterial challenge : time to react (p. 54). doi:10.2900/2518

European Medecines Agency (EMA). (2009b). Reflection paper on the use of third and four generation cephalosporins in food producing animals in the European Union: development of resistance and impact on human and animal health. *Journal Veterinary Pharmacology Therapy*, *32*, 515–533. doi: 10.1111/j.1365-2885.2009.01075.x.

European Medicine Agency (2012). Convenia: Annex I – Summary of product characteristics. London: EMA. Retrieved December, 5 from http://www.ema.europa.eu/

European Medicines Agency (EMA). (2013). Sales of veterinary antimicrobial agents in 19 EU/EEA countries in 2011 (EMA/88728/2012).

Falagas, M. E. & Karageorgopoulos, D. E. (2008). Pandrug resistance (PDR), extensive drug resistance (XDR), and multidrug resistance (MDR) among Gram-negative bacilli: need for international harmonization in terminology. *Clinical Infectious Diseases*, *46*, 1121–2. doi: 10.1086/528867

Fam, N., Gamal, D., Said, M. El., Defrawy, I. El, Dadei, E. El., Attar, S. El., Sorur, A., Ahmed, S. & Klena, J. (2013). Prevalence of Plasmid-Mediated amp C Genes in Clinical Isolates of Enterobacteriaceae from. *British Microbiology Research Journal*, *3*(4), 525–537

Farmer III, J., Boatwright, K. D. & Janda, J. (2008). Enterobacteriaceae: introduction and identification. In Murray, P. R., Baron, E.J., Jorgensen, J.H., Landry, M.L., Pfaller, M. (Eds.), *Manual of Clinical Microbiology* (pp. 649–669). Washington DC: ASM Press

Ferech, M., Coenen, S., Malhotra-Kumar, S., Dvorakova, K., Hendrickx, E., Suetens, C. & Goossens, H. (2006). European Surveillance of Antimicrobial Consumption (ESAC): outpatient antibiotic use in Europe. *Journal of Antimicrobial Chemotherapy*, *58*(2), 401–7. doi:10.1093/jac/dkl188

Feria, M. C. (2001). Infecção no tracto urinário no cão por Escherichia coli: Abordagem ao Diagnóstico, Fisiopatologia e resistência às β-lactaminas. Faculdade de Medicina Veterinária – Universidade Técnica de Lisboa

Feria, C.. Ferreira, E., Correia, J.D., Gonçalves, J. & Caniça, M. (2002). Patterns and mechanisms of resistance to beta-lactams and beta-lactamase inhibitors in uropathogenic Escherichia coli isolated from dogs in Portugal. *Journal of Antimicrobial Chemotherapy*, *49*(1), 77–85. doi:10.1093/jac/49.1.77

Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. (2005). Mobile Genetic Elements: the Agents of Open Source Evolution. *Nature Reviews. Microbiology*, *3*(9), 722–32. doi:10.1038/nrmicro1235

Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E. & Courvalin, P. (1997). Multidrug resistance in Yersinia pestis mediated by a transferable plasmid. *The New England Journal of Medicine*, *337*(10), 677–80. doi:10.1056/NEJM199709043371004

Garau, J. (1994). Beta-lactamases: current situation and clinical importance. *Intensive Care Medecine*, *S5-9*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7962990

García-Fernández, A., Chiaretto, G., Bertini, A., Villa, L., Fortini, D., Ricci, A. & Carattoli, A. (2008). Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum β-lactamases in Escherichia coli and Salmonella of human and animal origin. *Journal of Antimicrobial Chemotherapy*, *61*(6), 1229–33. doi:10.1093/jac/dkn131

German, A. J., Halladay, L. J. & Noble, P. J. (2010). First-choice therapy for dogs presenting with diarrhoea in clinical practice. *The Veterinary Record*, *167*(21), 810–814. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21262629

Giammanco, G., Grimont, P. A. D., Grimont, F. & Giammanco, G. M. (2011). Genotyping of the genus Proteus by rpoB sequence analysis. *Italian Journal of Public Health*, *61*(7), 1638–1644. doi:DOI: 10.2427/5992

Gillespie, S. H., Basu, S., Dickens, A. L., O'Sullivan, D. M. & McHugh, T. D. (2005). Effect of subinhibitory concentrations of ciprofloxacin on Mycobacterium fortuitum mutation rates. *Journal of Antimicrobial Chemotherapy*, *56*(2), 344–8. doi:10.1093/jac/dki191

González, J. M., Medrano, M, F. J., Costello, A. L., Tierney, D. L. & Vila, A. J. (2007). The Zn2 position in metallo-β-lactamases is critical for activity: a study on chimeric metal sites on a conserved protein scaffold. *Journal of Molecular Biology*, *373*(5), 1141–56. doi:10.1016/j.jmb.2007.08.031

Goossens, H., Ferech, M., Vander Stichele, R. & Elseviers, M. (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet*, *365*(9459), 579–87. doi:10.1016/S0140-6736(05)17907-0

Goyanes, M. J., Cercenado, E., Insa, R., Morente, A., Alcalá, L. & Bouza, E. (2007). High rates of antimicrobial co-resistance among Enterobacteriaceae : comparative analysis between clinical isolates resistant and susceptible to third-generation cephalosporins. *Revista Espanola de Quimioterapia*, *20*(N^{o} 2), 216–221. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17893759

Grave, K., Bangen, M., Engelstad, M. & Søli, N. E. (1992). Prescribing of veterinary and human preparations for animals in Norway. Was the preparation approved for the animal species for which it was prescribed. *Journal of Veterinary Pharmacology and Therapeutics*, *15*(1), 45–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1573705

Grimont, F. & Grimont, P. A. D. (2006). The Genus Serratia. In S. Falkow, E. Rosenberg, E. Stackebrandt (Eds), *Prokaryotes* (pp. 219–244). doi:10.1007/0-387-30746-x_11

Groisman, E. A. & Ochman, H. (1997). How Salmonella became a pathogen. *Trends in Microbiology*, *5*(9), 343–9. doi:10.1016/S0966-842X(97)01099-8

Guardabassi, L., Houser G.A.,Frank, L.A., Papich, M.G. (2008). Guidelines for antimicrobial use in dogs and cats. In L. Guardabassi, L. Jensen, H. Krusse, *Guide to Antimicrobial Use in Animals*, 183-206. Oxford: Blackwell Publishing

Guarner, F. & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet*, *361*(9356), 512–9. doi:10.1016/S0140-6736(03)12489-0

Gustafson, R. H. & Bowen, R. E. (1997). Antibiotic use in animal agriculture. *Journal of Applied Microbiology*, *83*(5), 531–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9418018

Haldorsen, B., Aasnaes, B., Dahl, K. H., Hanssen, A.M., Simonsen, G. S., Walsh, T. R., Sundsfjord, A. & Lundblad, E. W. (2008). The AmpC phenotype in Norwegian clinical isolates of Escherichia coli is associated with an acquired ISEcp1-like ampC element or hyperproduction of the endogenous AmpC. *Journal of Antimicrobial Chemotherapy*, *62*(4), 694–702. doi:10.1093/jac/dkn257

Hall, B. G. & Barlow, M. (2005). Revised Ambler classification of {beta}-lactamases. *Journal of Antimicrobial Chemotherapy*, *55*(6), 1050–1. doi:10.1093/jac/dki130

Hammerum, A. M. & Heuer, O. E. (2009). Human health hazards from antimicrobial-resistant Escherichia coli of animal origin. *Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America*, *48*(7), 916–21. doi:10.1086/597292

Hanson, N. D. (2003). AmpC β-lactamases: what do we need to know for the future? *Journal of Antimicrobial Chemotherapy*, *52*(1), 2–4. doi:10.1093/jac/dkg284

Hanson, N. D. & Sanders, C. C. (1999). Regulation of inducible AmpC β-lactamase expression among Enterobacteriaceae. *Current Pharmaceutical Design*, *5*(11), 881–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10539994

Hawkey, P. M. & Jones, A. M. (2009). The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*, *64 Suppl 1*(suppl_1), i3–10. doi:10.1093/jac/dkp256

Heritage, J., Gascoyne-binzi, D. & Hawkey, P. M. (1999). Evolution and spread of SHV extended-spectrum β-lactamases in Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy*, *44*, 309–318. doi:10.1093/jac/44.3.309

Honoré, N., Nicolas, M. H. & Cole, S. T. (1986). Inducible cephalosporinase production in clinical isolates of Enterobacter cloacae is controlled by a regulatory gene that has been deleted from Escherichia coli. *The EMBO journal*, *5*(13), 3709–14. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1167415/

Hopkins, K. L., Liebana, E., Villa, L., Batchelor, M., Threlfall, E. J. & Carattoli, A. (2006). Replicon Typing of Plasmids Carrying CTX-M or CMY β-Lactamases Circulating among Salmonella and Escherichia coli Isolates. *Antimicrobial Agents and Chemotherapy*, *50*(9), 3203–6. doi:10.1128/AAC.00149-06

Huang, X. Z., Frye, J. G., Chahine, M. A., Glenn, L. M., Ake, J. A., Su, W., S., Nikolich, M.P. & Lesho, E. P. (2012). Characteristics of plasmids in multi-drug-resistant Enterobacteriaceae isolated during prospective surveillance of a newly opened hospital in Iraq. *PloS one*, *7*(7), e40360. doi:10.1371/journal.pone.0040360

Hughes, L. A., Williams, N., Clegg, P., Callaby, R., Nuttall, T., Coyne, K., Pinchbeck, G. & Dawson, S. (2012). Cross-sectional survey of antimicrobial prescribing patterns in UK small animal veterinary practice. *Preventive Veterinary Medicine*, *104*(3-4), 309–16. doi:10.1016/j.prevetmed.2011.12.003

Hujer, A. M., Kania, M., Gerken, T., Anderson, V. E., Buynak, J. D., Ge, X., Caspers, P., Page, M.G.P., Rice, L. & Bonomo, R. A. (2005). Structure-Activity Relationships of Different β -Lactam Antibiotics against a Soluble Form of Enterococcus faecium PBP5 , a Type II Bacterial Transpeptidase. *Antimicrobial Agents and Chemotherapy*, *49*(2), 612–618. doi:10.1128/AAC.49.2.612

Hunter, S. B., Vauterin, P., Lambert-fair, M. A., Duyne, M. S. Van, Kubota, K., Graves, L., Wrigley, D., Barrett, T. & Ribot, E. (2005). Establishment of a Universal Size Standard Strain for Use with the PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocols : Converting the National Databases to the New Size Standard. *Journal of Clinical Microbiology*, *43*(3), 1045–1050. doi:10.1128/JCM.43.3.1045

Infarmed. (2012). *Prontuário Terapêutico - 11*. ISBN: 978-989-8369-11-6

Jacoby, G. A. (1994). Genetics of Extended-Spectrum beta-Lactamases. *European Journal of Clinical Microbiology & Infectious Diseases : official publication of the European Society of Clinical Microbiology*, *13 Suppl 1*, S2–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7821301

Jacoby, G. A. (2009). AmpC β-lactamases. *Clinical Microbiology Reviews*, *22*(1), 161–82, Table of Contents. doi:10.1128/CMR.00036-08

Johnson, T. J., Wannemuehler, Y. M., Johnson, S. J., Logue, C. M., White, D. G., Doetkott, C. & Nolan, L. K. (2007). Plasmid Replicon Typing of Commensal and Pathogenic Escherichia coli Isolates. *Applied and Environmental Microbiology*, *73*(6), 1976–83. doi:10.1128/AEM.02171-06

Jones, R. N. (1998). Important and Emerging β-Lactamase-mediated Resistances in Hospital-based Pathogens: The Amp C Enzymes. *Diagnostic Microbiology and Infectious Disease*, *31*(3), 461–466. doi:http://dx.doi.org/10.1016/S0732-8893(98)00029-7

Kaper, J. B., Nataro, J. P. & Mobley, H. L. (2004). Pathogenic Escherichia coli. *Nature Reviews*, *2*(2), 123–40. doi:10.1038/nrmicro818

Kaur, M. & Aggarwal, A. (2013). Occurrence of the CTX-M, SHV and the TEM Genes Among the Extended Spectrum β-Lactamase Producing Isolates of Enterobacteriaceae in a Tertiary Care Hospital of North India. *Journal of Clinical and Diagnostic Research*, *7*(4), 642–5. doi:10.7860/JCDR/2013/5081.2872

Kim, S. R. & Komano, T. (1997). The Plasmid R64 Thin Pilus Identified as a Type IV Pilus. *Journal of Bacteriology*, *179*(11), 3594–603. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=179153&tool=pmcentrez&rendertype=abstract

Knothe, H., Shah, P., Krcmery, V., Antal, M. & Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, and cefuroxime in clinical isolates of Klebsiella pneumoniae and Serratia marcescens. *Infection*, *7*, 315. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6321357

Kools, S. A. E., Moltmann, J. F. & Knacker, T. (2008). Estimating the use of veterinary medicines in the European union. *Regulatory Toxicology and Pharmacology*, *50*(1), 59–65. doi:10.1016/j.yrtph.2007.06.003

Lai, E., Birren, B.W., Clark, S.M., Simon, M.I., & Hood, L. (1989). Pulsed field gel electrophoresis. *Biotechniques*, *7*, 34–42.

Lan, R. & Reeves, P. R. (2002). Escherichia coli in disguise: molecular origins of Shigella. *Microbes and Infection*, *4*(11), 1125–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12361912

Lee, N, Yuen, K. Y. & Kumana, C. R. (2003). Clinical role of beta-lactam/beta-lactamase inhibitor combinations. *Drugs*, *63*(14), 1511–1524. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12834367

Li, X. Z., Mehrotra, M., Ghimire, S. & Adewoye, L. (2007). β-Lactam resistance and β-lactamases in bacteria of animal origin. *Veterinary Microbiology*, *121*(3-4), 197–214. doi:10.1016/j.vetmic.2007.01.015

Li, Y., Li, Q., Du, Y., Jiang, X., Tang, J., Wang, J., Li, G. & Jiang, Y. (2008). Prevalence of plasmid-mediated AmpC β-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-Type AmpC β-lactamase resistance in China. *Journal of Clinical Microbiology*, *46*(4), 1317–21. doi:10.1128/JCM.00073-07

Ligon, B. L. (2004). Penicillin: its discovery and early development. *Seminars in Pediatric Infectious Diseases*, *15*(1), 52–57. doi:10.1053/j.spid.2004.02.001

Linde, H. J., Neubauer, H., Meyer, H., Aleksic, S. & Lehn, N. (1999). Identification of Yersinia Species by the Vitek GNI card. *Journal of Clinical Microbiology*, *37*(1), 211–4. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC84211/

Lindsey, R. L., Fedorka-Cray, P. J., Frye, J. G. & Meinersmann, R. J. (2009). Inc A/C plasmids are prevalent in multidrug-resistant Salmonella enterica isolates. *Applied and Environmental Microbiology*, *75*(7), 1908–15. doi:10.1128/AEM.02228-08

Lister, P. D. (2006). The role of pharmacodynamic research in the assessment and development of new antibacterial drugs. *Biochemical Pharmacology, 71(7),* 1057-1065. doi:10.1016/j.bcp.2005.10.038

Livermore, D. M. & Woodford, N. (2006). The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. *Trends in Microbiology*, *14*(9), 413–420. doi:10.1016/j.tim.2006.07.008

Luria, S. E. (1947). Recent Advances in Bacterial Genetics. *Bacterial Review*, *11*(1), 1–40. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC440908/

MacGowan, A. P. & Wise, R. (2001). Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *Journal of Antimicrobial Chemotherapy*, *48*(suppl 1), 17–28. doi:10.1093/jac/48.suppl_1.17

Maddison, J. (2009). Making drug choices : rational antibacterial therapy. *Irish Veterinary Journal*, *62*(7), 469–475. Retrieved from http://www.veterinaryirelandjournal.com

Majiduddin, F. K., Materon, I. C. & Palzkill, T. G. (2002). Molecular analysis of beta-lactamase structure and function. *International Journal of Medical Microbiology*, *292*(2), 127–37. doi:10.1078/1438-4221-00198

Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN). (2007). Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2006/2007. Retrieved August 30, from http://www.wageningenur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/central-veterinary-institute/Publicaties-CVI/MARAN-Rapporten.htm

Martins da Costa, P., Loureiro, L. & Matos, A.J.F. (2013). Transfer of Multidrug-Resistant Bacteria Between Intermingled Ecological Niches: The Interface Between Humans, Animals and the Environment. *International Journal of Environmental Research and Public Health*, *10*(1), 278–94. doi:10.3390/ijerph10010278

Massova, I. & Mobashery, S. (1998). Kinship and Diversification of Bacterial Penicillin-Binding Proteins and β-Lactamases. *Antimicrobial Agents and Chemotherapy*, *42*(1), 1–17. Retrieved from http://aac.asm.org/content/42/1/1.full

Mata, C., Navarro, F., Miró, E., Walsh, T. R., Mirelis, B. & Toleman, M. (2011). Prevalence of SXT/R391-like integrative and conjugative elements carrying blaCMY-2 in Proteus mirabilis. *Journal of Antimicrobial Chemotherapy*, *66*(10), 2266–70. doi:10.1093/jac/dkr286

Mataseje, L. F., Baudry, P. J., Zhanel, G. G., Morck, D. W., Read, R. R., Louie, M. & Mulvey, M. R. (2010). Comparison of CMY-2 plasmids isolated from human, animal, and environmental Escherichia coli and Salmonella spp. from Canada. *Diagnostic Microbiology and Infectious Disease*, *67*(4), 387–91. doi:10.1016/j.diagmicrobio.2010.02.027

Mateus, A., Brodbelt, D. C., Barber, N. & Stärk, K. D. C. (2011). Antimicrobial usage in dogs and cats in first opinion veterinary practices in the UK. *Journal of Small Animal Practice*, *52*(10), 515–21. doi:10.1111/j.1748-5827.2011.01098.x

Matsumoto, Y., Ikeda, F., Kamimura, T., Yokota, Y. & Mine, Y. (1988). Novel Plasmid-Mediated β-Lactamase from Escherichia coli That Inactivates Oxyimino-Cephalosporins. *Antimicrobial Agents and Chemotherapy*, *32*(8). doi:10.1128/AAC.32.8.1243.Updated

Mayer, D. G. (2010). Genetic Regulatory Mechanisms, University of South Carolina School of Medicine. Retrieved July 15, from http://pathmicro.med.sc.edu/book/bact-sta.htm

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L. & Wilson, R. K. (2001). Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. *Nature*, *413*(6858), 852–6. doi:10.1038/35101614

Medeiros, A. A. (1997). Evolution and Dissemination of β-Lactamases Accelerated by Generations of β-Lactam Antibiotics. *Clinical Infectious Diseases*, *24*(Supplement 1), S19–S45. doi:10.1093/clinids/24.Supplement_1.S19

Moosdeen, F., Williams, J. D. & Yamabe, S. (1988). Antibacterial Characteristics of YTR 830, a Sulfone β-Lactamase Inhibitor, Compared with those of Clavulanic Acid and Sulbactam. *Antimicrobial Agents and Chemotherapy*, *32*(6), 925–7.

Morley, P. S., Apley, M. D., Besser,T. E., Burney, D. P. P., Fedorka-Cray, P., Papich, M., Traub-Dargatz, J. L. & Weese, J.S. (2005). Antimicrobial Drug Use in Veterinary Medicine: ACVIM Consensus Statement. *Journal of Veterinary Internal Medicine*, *19*, 617–629.

Murphy, C.P., Reid-Smith, R. J., Boerlin, P., Weese, J.S, Prescott, J. F., Janecko, N. & McEwen, S. A. (2012). Out-patient antimicrobial drug use in dogs and cats for new disease events from community companion animal practices in Ontario. *The Canadian Veterinary Journal*, *53*(3), 291–298. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22942447

Naas, T., Mikami, Y., Imai, T., Poirel, L. & Bacte, S. De. (2001). Characterization of In53 , a Class 1 Plasmid- and Composite Transposon-Located Integron of Escherichia coli Which Carries an Unusual Array of Gene Cassettes. *Journal of Bacteriology*, *183*(1), 235–249. doi:10.1128/JB.183.1.235

Naseer, U., Haldorsen, B., Simonsen, G. S. & Sundsfjord, A. (2010). Sporadic occurrence of CMY-2-producing multidrug-resistant Escherichia coli of ST-complexes 38 and 448, and ST131 in Norway. *Clinical Mcrobiology and Infection*, *16*(2), 171–8. doi:10.1111/j.1469-0691.2009.02861.x

Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic Escherichia coli. *Clinical Microbiology Reviews*, *11*(1), 142–201. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9457432

Nataro, J., Bopp, C.A., Fields, P.I., Kaper, J.B. & Stockbine, N. A. (2008). Escherichia, Shigella, and Salmonella. In Murray, P. R., Baron, E.J., Jorgensen, J.H., Landry, M.L., Pfaller, M. (Eds.). *Manual of Clinical Microbiology* (pp. 670–683). Washington DC: ASM Press

Nelson, E. C. & Elisha, B. G. (1999). Molecular Basis of AmpC Hyperproduction in Clinical Isolates of Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *43*(4), 957–9. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC89235/

Nordmann, P., Dortet, L. & Poirel, L. (2012). Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends in Molecular Medicine*, *18*(5), 263–72. doi:10.1016/j.molmed.2012.03.003

O'Hara, C. M., Brenner, F. W. & Miller, J. M. (2000). Classification, Identification, and Clinical Significance of Proteus, Providencia, and Morganella. *Clinical Microbiology Reviews*, *13*(4), 534–46. Retrieved from http://cmr.asm.org/content/13/4/534.full

O'Keefe, A., Hutton, T. A., Schifferli, D. M. & Rankin, S. C. (2010). First Detection of CTX-M and SHV Extended-Spectrum β-Lactamases in Escherichia coli Urinary Tract Isolates from Dogs and Cats in the United States. *Antimicrobial Agents and Chemotherapy*, *54*(8), 3489–92. doi:10.1128/AAC.01701-09

Olsson, O., Bergström, S., Lindberg, F. P. & Normark, S. (1983). ampC β-lactamase hyperproduction in Escherichia coli: natural ampicillin resistance generated by horizontal chromosomal DNA transfer from Shigella. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(24), 7556–60. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC534379/

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A. & Bonomo, R. A. (2011). Carbapenems: past, present, and future. *Antimicrobial Agents and Chemotherapy*, *55*(11), 4943–60. doi:10.1128/AAC.00296-11

Passantino, A. (2007). Ethical aspects for veterinarians regarding antimicrobial drug use in Italy. *International Journal of Antimicrobial Agents*, *29*(3), 240–4. doi:10.1016/j.ijantimicag.2006.09.023

Paterson, D. L. & Bonomo, R. A. (2005). Extended-Spectrum β-Lactamases: a Clinical Update. *Clinical Microbiology Reviews*, *18*(4), 657–686. doi:10.1128/CMR.18.4.657

Paterson, D. L. (2006). Resistance in gram-negative bacteria: Enterobacteriaceae. *American Journal of Infection Control, 34*(5), S20-S28. doi: 10.1016/j.ajic.2006.05.238

Pedersen, K., Pedersen, K., Jensen, H., Finster, K., Jensen, V. F. & Heuer, O. E. (2007). Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. *Journal of Antimicrobial Chemotherapy*, *60*(4), 775–81. doi:10.1093/jac/dkm269

Peláez, F. (2006). The historical delivery of antibiotics from microbial natural products--can history repeat? *Biochemical Pharmacology*, *71*(7), 981–90. doi:10.1016/j.bcp.2005.10.010

Pérez-Pérez, F. J. & Hanson, N. D. (2002). Detection of Plasmid-Mediated AmpC β-Lactamase Genes in Clinical Isolates by Using Multiplex PCR, *40*(6), 2153–2162. doi:10.1128/JCM.40.6.2153

Pfeifer, Y., Cullik, A. & Witte, W. (2010). Resistance to cephalosporins and carbapenems in gram-negative bacterial pathogens. *International Journal of Medical Microbiology*, *300*(6), 371–9. doi:10.1016/j.ijmm.2010.04.005

Pitout, J. D., Sanders, C. C. & Sanders, W. E. (1997). Antimicrobial resistance with focus on β-lactam resistance in gram-negative bacilli. *The American Journal of Medicine*, *103*(1), 51–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9236486

Pitout, J. D., Nordmann, P., Laupland, K. B. & Poirel, L. (2005). Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *Journal of Antimicrobial Chemotherapy*, *56*(1), 52–9. doi:10.1093/jac/dki166

Pitout, J. D. & Laupland, K. B. (2008). Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infectious Disease*, *8*(March), 159–166. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18291338

Pitout, J. D. (2008a). Multiresistant Enterobacteriaceae: new threat of an old problem. *Expert Review of Anti -Infective Therapy*, *6*, 657–69. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18847404

Podschun, R. & Ullmann, U. (1998). Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clinical Microbiology Reviews*, *11*(4), 589–603. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9767057

Pomba, C., Mendonça, N., Costa, M., Louro, D., Baptista, B., Ferreira, M., Correia, J.D. & Caniça, M. (2006). Improved multiplex PCR method for the rapid detection of β-lactamase genes in Escherichia coli of animal origin. *Diagnostic Microbiology & Infectious Disease*, *56*(1), 103–106. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16678992

Poole, K. (2004). Resistance to β-lactam antibiotics. *Cellular and Molecular Life*, *61*(17), 2200–23. doi:10.1007/s00018-004-4060-9

Queenan, A. M. & Bush, K. (2007). Carbapenemases: the versatile beta-lactamases. *Clinical Microbiology Reviews*, *20*(3), 440–58, table of contents. doi:10.1128/CMR.00001-07

Rantala, M., Hölsö, K., Lillas, A, Huovinen, P. & Kaartinen, L. (2004). urvey of condition-based prescribing of antimicrobial drugs for dogs at a veterinary teaching hospital. *The Veterinary Record*, 155(9), 259–262. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15461362

Reading, C. & Cole, M. (1977). Clavulanic acid: a beta-lactamase-inhiting beta-lactam from Streptomyces clavuligerus. *Antimicrobial Agents and Chemotherapy*, *11*(5), 852–7. Retrieved from http://aac.asm.org/content/11/5/852

Regula, G., Torriani, K., Gassner, B., Stucki, F. & Müntener, C. R. (2009). Prescription patterns of antimicrobials in veterinary practices in Switzerland. *Journal of Antimicrobial Chemotherapy*, *63*(4), 805–11. doi:10.1093/jac/dkp009

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B. & Barrett, T.J. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. *Foodborn Pathogens and Disease*, *3*(1), 59–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16602980

Rice, L. B. (1998). Tn 916 Family Conjugative Transposons and Dissemination of Antimicrobial Resistance Determinants. *Antimicrobial Agents and Chemotherapy*, *42*(8), 1871–1877. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9687377

Rizzi, A., Pontiroli, A., Brusetti, L., Borin, S., Sorlini, C., Abruzzese, A., … Daffonchio, D. (2008). Strategy for In Situ Detection of Natural Transformation-Based Horizontal Gene Transfer Events. *Applied and Environmental Microbiology*, *74*(4), 1250–4. doi:10.1128/AEM.02185-07

Roberts, D. E., McClain, H. M., Hansen, D. S., Currin, P. & Howerth, E. W. (2000). An Outbreak of Klebsiella Pneumoniae Infection in Dogs with Severe Enteritis and Septicemia. *Journal of Veterinary Diagnostic Investigation*, *12*(2), 168–173. doi:10.1177/104063870001200215

Rodloff, A., Bauer, T., Ewig, S., Kujath, P. & Müller, E. (2008). Susceptible, intermediate, and resistant - The Intensity of Antibiotic Action. *Deutsches Ärzteblatt International*, *105*(39), 657–62. doi:10.3238/arztebl.2008.0657

Rolain, J. (2013). Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Frontiers in Microbiology 24*(4), 173. doi: 10.3389/fmicb.2013.00173

Rossolini, G. M., D'Andrea, M. M. & Mugnialoi, C. (2008). The spread of CTX-M-type extended-spectrum beta-lactamases *Clinical Microbiology and Infectioun*, *14*(1), 33–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18154526

Rowe-Magnus, D. A. & Mazel, D. (2002). The role of integrons in antibiotic resistance gene capture. *International Journal of Medical Microbiology :*, *292*(2), 115–25. doi:10.1078/1438-4221-00197

Rupp, M. E. & Fey, P. D. (2003). Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae: considerations for diagnosis, prevention and drug treatment. *Drugs*, *63*(4), 353–65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12558458

Sanchez, S., Stevenson, M. A. M., Hudson, C. R., Maier, M., Buffington, T., Dam, Q. & Maurer, J. J. (2002). Characterization of Multidrug-Resistant Escherichia coli Isolates Associated with Nosocomial Infections in Dogs. *Journal of Clinical Microbiology*, *40*(10), 3586–3595. doi:10.1128/JCM.40.10.3586

Sanders, W. E. & Sanders, C. C. (1997). Enterobacter spp.: Pathogens Poised to Flourish at the Turn of the Century. *Clinical Microbiology Reviews*, *10*(2), 220–41. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC172917/

Saxon, A., Hassner, A., Swabb, E.A., Wheeler, B. & Adkinson, N. F. (1984). Lack of cross-reactivity between aztreonam , a monobactam antibiotic, and penicillin in penicillin-allergic subjects. *Journal of Infectious Disease*, *149*(1), 16–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6537963

Schmidtke, A. J. & Hanson, N. D. (2006). Model system to evaluate the effect of ampD mutations on AmpC-mediated beta-lactam resistance. *Antimicrobial Agents and Chemotherapy*, *50*(6), 2030–7. doi:10.1128/AAC.01458-05

Seiffert, S. N., Hilty, M., Perreten, V. & Endimiani, A. (2013). Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: An emerging problem for human health? *Drug resistance updates*, *16*(1-2), 22–45. doi:10.1016/j.drup.2012.12.001

Shea, A., Mccarthy, R. & Lindenmayer, J. (2012). Therapeutic Antibiotic Use Patterns in Dogs: Observations from a Veterinary Teaching Hospital. *Journal of Small Animal Pratice*, *52*(6), 310–318. doi:10.1111/j.1748-5827.2011.01072.x

Sherley, M., Gordon, D.M. & Collignon, P. J. (2004). Species differences in plasmid carriage in the Enterobacteriaceae. *Plasmid*, *49*(1), 79–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12584004

Shih, C. C., Chen, Y. C., Chang, S. C., Luh, K. T. & Hsieh, W. C. (1996). Bacteremia Due to Citrobacter species: Significance of Primary Intraabdominal Infection. *Clinical Infectious Diseases*, *23*(3), 543–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8879778

Singtohin, S., Chanawong, A., Lulitanond, A., Sribenjalux, P., Auncharoen, A., Kaewkes, W., Songsri, J. & Pienthaweechai K. (2010). CMY-2, CMY-8b, and DHA-1 plasmid-mediated AmpC β-lactamases among clinical isolates of Escherichia coli and Klebsiella pneumoniae from a university hospital, Thailand. *Diagnostic Microbiology and Infectious Disease*, *68*(3), 271–277. doi: 10.1016/j.diagmicrobio.2010.06.014

Siu, L. K., Lu, P., Chen, J., Lin, F. M. & Chang, S. (2003). High-Level Expression of AmpC β -Lactamase Due to Insertion of Nucleotides between -10 and -35 Promoter Sequences in Escherichia coli Clinical Isolates : Cases Not Responsive to Extended-Spectrum-Cephalosporin Treatment. *Antimicrobial agents and chemotherapy*, *47*(7), 2138–2144. doi:10.1128/AAC.47.7.2138

Smet, A., Martel, A., Persoons, D., Dewulf J, Heyndrickx, M., Herman L, Haesebrouck, F. & Butaye ,P. (2010). Broad-spectrum β-lactamases among Enterobacteriaceae of animal origin: molecular aspects, mobility and impact on public health. *FEMS Microbiolgy Review*, *34*(3), 295–316. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20030731

Stamm, I., Hailer, M., Depner, B., Kopp, P. A. & Rau, J. (2013). Yersinia enterocolitica in diagnostic fecal samples from European dogs and cats: identification by fourier transform infrared spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology*, *51*(3), 887–93. doi:10.1128/JCM.02506-12

Starr, M. P. & Reynolds, D. M. (1951). Streptomycin Resistance of Coliform Bacteria from Turkeys Fed Streptomycin. *American Journal of Public Health. Clinical Microbiology Reviews*, *41*, 1375–1380. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1525794/

Stegemann, M. R., Passmore, C. A., Sherington, J., Lindeman, C. J., Papp, G., Weigel, D. J. & Skogerboe, T. L. (2006). Antimicrobial activity and spectrum of cefovecin, a new extended- spectrum cephalosporin, against pathogens collected from dogs and cats in Europe and North America. *Antimicrobial Agents and Chemotherapy*, *50*(7), 2286–92. doi:10.1128/AAC.00077-06

Stock, J. B., Rauch, B. & Roseman, S. (1977). Periplasmic space in Salmonella typhimurium and Escherichia coli. *Journal of Biological Chemistry.*, *252*(21), 7850–7861. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/334768

Stokes, H. W. & Gillings, & M. R. (2011). Gene flow,mobile genetic elements and the recruitment of of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiology Review*, *35*(5), 790–819. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21517914

Struve, C. & Krogfelt, K. A. (2004). Pathogenic potential of environmental Klebsiella pneumoniae isolates. *Environmental Microbiology*, *6*(6), 584–90. doi:10.1111/j.1462-2920.2004.00590.x

Swedish Veterinary Association. (2009). *Guidelines for the clinical use of antibiotics in the treatment of dogs and cats*. Stockholm: SVA. Retrieved March 20, from http://svf.se/Documents/S%C3%A4llskapet/Sm%C3%A5djurssektionen/Policy%20ab%20english%2010b.pdf

SWEDRES-SVARM 2012. Use of antimicrobials and occurrence of antimicrobial resistance in Sweden. Solna/Uppsala ISSN 1650-6332

Sykes, R. B., Bonner, D. P., Bush, K., Georgopapadakou, N. H. & Wells, J. S. (1981). Monobactams--monocyclic -lactam antibiotics produced by bacteria. *Journal of Antimicrobial Chemotherapy*, *8*(suppl E), 1–16. doi:10.1093/jac/8.suppl_E.1

Tamang, M. D., Nam, H.-M., Jang, G.-C., Kim, S.-R., Chae, M. H., Jung, S.-C., Byun, L.-W., Park, Y.H. & Lim, S.-K.(2012). Molecular Characterization of Extended-Spectrum-β-Lactamase-Producing and Plasmid-Mediated AmpC β-Lactamase-Producing Escherichia coli Isolated from Stray Dogs in South Korea. *Antimicrobial Agents and Chemotherapy*, *56*(5), 2705–12. doi:10.1128/AAC.05598-11

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *American Journal of Medicine*, *119*(6 Suppl 1), S3–10; discussion S62–70. doi:10.1016/j.amjmed.2006.03.011

Thomson, K. S. & Smith Moland, E. (2000). Version 2000: the new β-lactamases of Gram-negative bacteria at the dawn of the new millennium. *Microbes and Infection*, *2*(10), 1225–1235. doi:10.1016/S1286-4579(00)01276-4

Thomson, K. S. (2001). Controversies about extended-spectrum and AmpC beta-lactamases. *Emerging Infectious Diseases*, *7*(2), 333–6. doi:10.3201/eid0702.700333

Thomson, K. H., Rantala, M. H. J., Viita-Aho, T. K., Vainio, O. M. & Kaartinen, L. A. (2009). Condition-based use of antimicrobials in cats in Finland: results from two surveys. *Journal of Feline Medicine and Surgery*, *11*(6), 462–6. doi:10.1016/j.jfms.2008.10.005

Tipper, D. & Strominger, J.L. (1965). Mechaninm of Action of Penicillins: A Proposal Based on their Structural Similarity to AcylL-D-Alanyl-D-Alanine. *Proceedings of the National Academy of Sciences of the United States of America*, *54*(4), 1133-1141. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC219812/

Todar, K. (2008). Todar´s Online Textbook of Bacteriology. Madison- Wisconsin. Retrieved from www.textbookofbacteriology.net

Trott, D. (2012). β-lactam Resistance in Gram-negative Pathogens Isolated from Animals. Current Pharmaceutical Design, 19*(2),* 239-49. doi.org/10.2174/138161213804070339

Ungemach, F. R., Müller-Bahrdt, D. & Abraham, G. (2006). Guidelines for prudent use of antimicrobials and their implications on antibiotic usage in veterinary medicine. *International Journal of Medical Microbiology*, *296 Suppl*, 33–8. doi:10.1016/j.ijmm.2006.01.059

Van den Bogaard, A. E. & Stobberingh, E. E. (2000). Epidemiology of resistance to antibiotics. Links between animals and humans. *International Journal of Antimicrobial Agents*, *14*(4), 327–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10794955

Vander Stichele, R. H., Elseviers, M. M., Ferech, M., Blot, S. & Goossens, H. (2006). Hospital consumption of antibiotics in 15 European countries: results of the ESAC Retrospective Data Collection (1997-2002). *Journal of Antimicrobial Chemotherapy*, *58*(1), 159–67. doi:10.1093/jac/dkl147

Vanni, M., Tognetti, R., Pretti, C., Crema, F., Soldani, G., Meucci, V. & Intorre, L. (2009). Antimicrobial susceptibility of Staphylococcus intermedius and Staphylococcus schleiferi isolated from dogs. *Research in Veterinary Science*, *87*(2), 192–5. doi:10.1016/j.rvsc.2009.01.011

Villa, L., García-Fernández, A., Fortini, D. & Carattoli, A. (2010). Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. *Journal of Antimicrobial Chemotherapy*, *65*(12), 2518–29. doi:10.1093/jac/dkq347

Veterinary Medical Doctorate (VMD). (2012). *Sales of Antimicrobial Products Authorised for Use as Veterinary Medicines in the UK in 2011*. Retrieved from http://www.vmd.defra.gov.uk/pdf/salesanti11.pdf

Watson, A.D. & Maddison, J. (2001). Systemic antibacterial drug use in dogs in Australia. *Australian Veterinary Journal*, *79*(11), 740–746. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11789908

Weese, J. S. (2006). Investigation of antimicrobial use and the impact of antimicrobial use guidelines in a small animal veterinary teaching hospital: 1995-2004. *Journal of the American Veterinary Medical Association*, *228*(4), 553–8. doi:10.2460/javma.228.4.553

Welch, T. J., Fricke, W. F., McDermott, P. F., White, D. G., Rosso, M.-L., Rasko, D. A, … Ravel, J. (2007). Multiple antimicrobial resistance in plague: an emerging public health risk. *PloS one*, *2*(3), e309. doi:10.1371/journal.pone.0000309

Westropp. J.L. (2008). Feline Idiopathic Cystitis: Pathophysiology and management. *Proceedings of the 33rd World Small Animal Congress 2008* – Dublin, Ireland, 294-295. doi:10.1016/j.cvsm.2004.03.002

Wilke, M. S., Lovering, A. L. & Strynadka, N. C. J. (2005). β-lactam antibiotic resistance: a current structural perspective. *Current Opinion in Microbiology*, *8*(5), 525–33. doi:10.1016/j.mib.2005.08.016

Winokur, P. L., Brueggemann, A., DeSalvo, D. L., Hoffmann, L., Apley, M. D., Uhlenhopp, E. K., … Doern, G. V. (2000). Animal and Human Multidrug-Resistant, Cephalosporin-Resistant Salmonella Isolates Expressing a Plasmid-Mediated CMY-2 AmpC β-Lactamase. *Antimicrobial Agents and Chemotherapy*, *44*(10), 2777–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10991860

Winokur, P. L., Vonstein, D. L., Hoffman, L. J. & Uhlenhopp, E. K. (2001). Evidence for Transfer of CMY-2 AmpC β-Lactamase Plasmids between Escherichia coli and Salmonella Isolates from Food Animals and Humans. *Antimicrobial Agents and Chemotherapy*, *45*(10), 2716–2722. doi:10.1128/AAC.45.10.2716

Wise, R., Hart, T. & Sprenger, M. (1998). Antimicrobial resistance Is a major threat to public health. *British Medical Journal*, *317*(September), 609–610. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1113826/

Woodford, N, Ellington, M.J. (2007). The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection*, *13*(1), 5–18. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17184282

World Organization for Animal Health (OIE). (2007). *List of Antimicrobials of Veterinary Importance*. Retrieved August 30, from http://web.oie.int/downld/Antimicrobials/OIE_list_antimicrobials.pdf

World Health Organization (WHO). (2007). *Critically important antimicrobials for Human medicine: Categorization for the development of risk management strategies to contain antimicrobial resistance due to non-Human antimicrobial Use: Report of the 2nd WHO expert meeting Copenhagen, Denmark*. ISBN 978 92 4 159574 2

World Health Organization (WHO). (2009). *Collaborating Centre for Drug Statistics and Methodology.*Retrieved August 5, from http://www.whocc.no/ddd/

World Health Organization (WHO). (2011). *Tackling antibiotic resistance from a food safety perspective in Europe* (p. 88). Europe. ISBN 978 92 890 1421 2

Wozniak, R. A. F. & Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nature Reviews. Microbiology*, *8*(8), 552–63. doi:10.1038/nrmicro2382

Yan, J., Ko, W., Jung, Y., Chuang, C. L. & Wu, J.J. (2002). Emergence of Klebsiella pneumoniae Isolates Producing Inducible DHA-1 β- Lactamase in a University Hospital in Taiwan. *Journal of Clinical Microbiology*, *40*(9), 3121–3126. doi:10.1128/JCM.40.9.3121

Zhanel, G.G., Wiebe, R., Dilay, L, Thomson, K., Rubinstein, E., Hoban, D.J,, Noreddin, A.M. & Karlowsky, J.A. (2007). Comparative review of the carbapenems. *Drugs*, *67*(7), 1027–1052. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17488146

Zhang, Y., Yakrus, M. A., Graviss, E. A., Williams-bouyer, N., Turenne, C., Kabani, A. & Wallace, R. J. (2004). Pulsed-Field Gel Electrophoresis Study of Mycobacterium abscessus Isolates Previously Affected by DNA Degradation. *Journal of Clinical Microbiology*, *42*(12), 5582–5587. doi:10.1128/JCM.42.12.5582

Zhao, S., White, D. G., Mcdermott, P. F., Friedman, S., English, L., Ayers, S.,Meng, J., Maurer, J. J., Holland, R. & Walker, R.D. (2001). Identification and Expression of Cephamycinase bla CMY Genes in Escherichia coli and Salmonella Isolates from Food Animals and Ground Meat. *Antimicrobial Agents and Chemotherapy*, *45*(12), 3647–3650. doi:10.1128/AAC.45.12.3647

Annex 1 – Critically important antimicrobial classes in veterinary and Human Medicine (adapted from Mateus *et al*., 2011; World Organization for animal Health [OIE], 2007; WHO, 2007).

Critically important antimicrobial classes	
Veterinary	Human
Aminoglycosides	Aminoglycosides
Cephalosporins (first to fourth generation)	Carbapenems
Macrolides	Cephalosporins (first to fourth generation)
Penicillins	Glycopeptides
Fluoroquinolones	Macrolides
Sulfonamides	Oxazolidinones
Tetracyclines	Penicillins
	Quinolones
	Streptogramins
	Tetracyclines
	Drugs for mycobacterial infections

Annex 2 – Characteristics of CMY and CTX-M β-lactamases and their encoding genes increasingly reported in animal-derived bacteria (adapted from Li, X.-Z., Mehrotra, Ghimire, & Adewoye, 2007).

	CMY	CTX-M
Bacterial species	*E. coli* and *Salmonella* spp.	*E. coli, Salmonella* spp. and other enteric Gram-negative bacteria
Gene location	Plasmid-borne	Plasmid-borne
Genetic association	Often associated genetically with other antimicrobial resistance determinants and transposons/integrons	Often coexisted with blaTEM-1 or derivatives and/or associated with other antimicrobial resistance determinants and transposons/integrons
Molecular class	Class C	Class A
Functional group	Group 1	Group 2be
ESBL	NO	YES
Substrate profile	Penicillin's and cephalosporins including cephamycins and oxyimino-cephalosporins. Not include carbapenems	Penicillin's, cephalosporins including oxyiminocephalosporins, and monobactams (aztreonam). Not include cephamycins and carbapenems
Inhibitor profile	Inhibited by aztreonam and/or cloxacillin, but not by clavulanate	Inhibited by clavulanate, sulbactam and tazobactam

Annex 3 – Survey - Antimicrobial use in companion animals.

Dia Europeu dos Antibióticos - Iniciativa da UE no domínio da saúde

Todos os anos, a **18 de Novembro**, celebra-se o **Dia Europeu dos Antibióticos**. Relembram-se os riscos associados ao uso inadequado de antibióticos e de informação sobre o uso racional dos mesmos.

A OMV associa-se a esta iniciativa da União Europeia pedindo a colaboração a todos os seus membros no estudo "**Utilização de Antibióticos em Animais de Companhia em Portugal**".

Este estudo está a ser efetuado na Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa e conta com a colaboração da OMV. Tem por objeto determinar os hábitos de prescrição de antibióticos dos Médico-Veterinários a exercer Clínica de animais de companhia em Portugal.

Por favor, clique no link e preencha o inquérito on-line!
https://www.surveymonkey.com/s/Y59WD96

A sua **contribuição** é **MUITO IMPORTANTE** para **melhorar o uso racional dos Antibióticos**.

Muito obrigada

Dia Europeu dos Antibióticos – 18 de Novembro.
A OMV associa-se a esta iniciativa da União Europeia pedindo a colaboração a todos os seus membros no estudo:

Utilização de antibióticos em animais de companhia, em Portugal.

OBJETIVO: através de um questionário sucinto para clínicos Veterinários obter informações no que respeita à utilização de antibióticos em animais de companhia Portugal. O tempo estimado necessário para completar este questionário é de aproximadamente 10 minutos. Todos os dados recolhidos no questionário serão tratados de forma confidencial e anónima.

O presente questionário apresenta 3 seções. A seção A consiste em informação geral sobre o Médico Veterinário; a seção B compreende a caracterização da prescrição de antibióticos; a seção C, consiste em determinar qual o antibiótico utilizado para determinada doença.

*Os inquiridos que pretendam ser informados sobre os resultados e conclusões do estudo deverão para o efeito indicar o endereço de correio eletrónico.

A- Informação Geral

1- Idade (anos):

2- Sexo:

- ○ Masculino
- ○ Feminino

3- A sua atividade profissional é exercida em:

- ○ Consultório Veterinário
- ○ Clínica Veterinária
- ○ Hospital Veterinário
- ○ Outro, por favor, especifique:

4- Onde exerce?

Localidade:
Concelho:

5- Há quanto tempo exerce a profissão (anos):

6- Possui especialização ou pós graduação:

- ○ Sim. Em que área?
- ○ Não

7- Endereço de correio eletrónico:*

B- Caraterização do uso de antibióticos em Centros de atendimento Médico-Veterinário

1 - Qual a espécie animal que se apresenta mais frequentemente a consultas:

- ○ Cão
- ○ Gato
- ○ Outra, por favor, especifique:

2- Com que regularidade prescreve antibióticos num dia normal no seu trabalho:

- ○ Sempre
- ○ Frequentemente
- ○ Algumas vezes
- ○ Raramente
- ○ Nunca

3- Com que frequência prescreve antibióticos de forma empírica, sem diagnóstico confirmado:

- ○ Sempre
- ○ Frequentemente
- ○ Algumas vezes
- ○ Raramente
- ○ Nunca

4- Qual a frequência com que realiza cultura bacteriana e testes de suscetibilidade antes da prescrição de antibióticos:

- ○ Sempre
- ○ Frequentemente
- ○ Algumas vezes
- ○ Raramente
- ○ Nunca

5- Com que frequência pesa os animais antes da prescrição de antibióticos:

- ○ Sempre
- ○ Frequentemente
- ○ Algumas vezes
- ○ Raramente
- ○ Nunca

6- Qual a sua via preferencial para administração de antibióticos em cães: (escolha 1 opção)

- ○ Oral
- ○ Injetável
- ○ Tópica

7- Qual a sua via preferencial para administração de antibióticos em gatos: (escolha 1 opção)

- ○ Oral
- ○ Injetável
- ○ Tópica

8- Indique os cinco antibióticos que prescreve com maior frequência para cães: (1-mais frequente; 5-menos frequente)

- ○ Penicilina G
- ○ Ampicilina
- ○ Amoxicilina
- ○ Amoxicilina/ Ácido clavulânico
- ○ Cefalexina
- ○ Cefovecina
- ○ Enrofloxacina
- ○ Marbofloxacina
- ○ Pradofloxacina
- ○ Doxiciclina
- ○ Gentamicina
- ○ Clindamicina
- ○ Trimetropim-sulfonamida
- ○ Metronidazol
- ○ Outro, por favor especifique:

9- Indique os cinco antibióticos que prescreve com maior frequência para gatos: (1-mais frequente; 5-menos frequente)

- ○ Penicilina G
- ○ Ampicilina
- ○ Amoxicilina
- ○ Amoxicilina/Ácido clavulânico
- ○ Cefalexina
- ○ Cefovecina
- ○ Enrofloxacina
- ○ Marbofloxacina
- ○ Pradofloxacina
- ○ Doxiciclina
- ○ Gentamicina
- ○ Clindamicina
- ○ Trimetropim-sulfonamida
- ○ Metronidazol
- ○ Outro, por favor especifique:

10- Assinale qual /quais os fatores que considera aquando da escolha do antibiótico:

- ☐ Características do dono (económicas, *compliance*)
- ☐ Caraterísticas e comportamento do animal
- ☐ Modo de ação do antibiótico, se é bacteriostático ou bactericida
- ☐ Farmacocinética/ Farmacodinâmica
- ☐ Espectro de ação
- ☐ Via de administração
- ☐ Baseado em testes de suscetibilidade
- ☐ Infeção aguda/crónica
- ☐ Agente patogénico envolvido
- ☐ Experiência clínica
- ☐ Opinião de colegas
- ☐ Outros, se sim especifique: _____

Para complementar este estudo, e obter informação mais detalhada sobre os hábitos de prescrição de antibióticos, agradecíamos que indicasse para as doenças referidas a seguir qual ou quais os antibióticos que prescreve com regularidade.

C- Caraterização da prescrição de antibióticos relativos às doenças mais prevalentes

11- Selecione para cada doença qual ou quais os antibióticos, que utiliza com maior frequência

11.1- Doenças de pele

Doença / Antibiótico	Piodermite superficial	Piodermite profunda	Abcesso/Trauma		Otite bacteriana	
	CÃO	**CÃO**	**CÃO**	**GATO**	**CÃO**	**GATO**
Penicilina G	○	○	○	○	○	○
Ampicilina	○	○	○	○	○	○
Amoxicilina	○	○	○	○	○	○
Amoxicilina/Ácido clavulânico	○	○	○	○	○	○
Cefalexina	○	○	○	○	○	○
Cefovecina	○	○	○	○	○	○
Enrofloxacina	○	○	○	○	○	○
Marbofloxacina	○	○	○	○	○	○
Pradofloxacina	○	○	○	○	○	○
Doxiciclina	○	○	○	○	○	○
Gentamicina	○	○	○	○	○	○
Clindamicina	○	○	○	○	○	○
Trimetropim-sulfonamida	○	○	○	○	○	○
Metronidazol	○	○	○	○	○	○
Florfenicol	○	○	○	○	○	○
Não uso terapêutica	○	○	○	○	○	○

11- Selecione para cada doença qual ou quais os antibióticos, que utiliza com maior frequência:
11.2- Aparelho Génito-urinário

Doença / Antibiótico	Infeção do trato urinário		ITU recorrente		Pielonefrite		Cistite idiopática felina	Prostatite	Piómetra
	CÃO	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**	**GATO**	**CÃO**	**CÃO**
Penicilina G	○	○	○	○	○	○	○	○	○
Ampicilina	○	○	○	○	○	○	○	○	○
Amoxicilina	○	○	○	○	○	○	○	○	○
Amoxicilina/ Ácido clavulânico	○	○	○	○	○	○	○	○	○
Cefalexina	○	○	○	○	○	○	○	○	○
Cefovecina	○	○	○	○	○	○	○	○	○
Enrofloxacina	○	○	○	○	○	○	○	○	○
Marbofloxacina	○	○	○	○	○	○	○	○	○
Pradofloxacina	○	○	○	○	○	○	○	○	○
Doxiciclina	○	○	○	○	○	○	○	○	○
Gentamicina	○	○	○	○	○	○	○	○	○
Clindamicina	○	○	○	○	○	○	○	○	○
Trimetropim-sulfonamida	○	○	○	○	○	○	○	○	○
Metronidazol	○	○	○	○	○	○	○	○	○
Nitrofurantoina*	○	○	○	○	○	○	○	○	○
Fosfomicina*	○	○	○	○	○	○	○	○	○
Não uso terapêutica	○	○	○	○	○	○	○	○	○

- Utilização ao abrigo da cascata.

11- Selecione para cada doença qual ou quais os antibióticos, que utiliza com maior frequência:
11.3- Aparelho Gastrointestinal

Doença / Antibiótico	Estomatite	Doença periodontal		Gastrite crónica		Enterite		Colite	
	GATO	**CÃO**	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**
Penicilina G	○	○	○	○	○	○	○	○	○
Ampicilina	○	○	○	○	○	○	○	○	○
Amoxicilina	○	○	○	○	○	○	○	○	○
Amoxicilina/ Ácido clavulânico	○	○	○	○	○	○	○	○	○
Cefalexina	○	○	○	○	○	○	○	○	○
Cefovecina	○	○	○	○	○	○	○	○	○
Enrofloxacina	○	○	○	○	○	○	○	○	○
Marbofloxacina	○	○	○	○	○	○	○	○	○
Pradofloxacina	○	○	○	○	○	○	○	○	○
Doxiciclina	○	○	○	○	○	○	○	○	○
Gentamicina	○	○	○	○	○	○	○	○	○
Clindamicina	○	○	○	○	○	○	○	○	○
Trimetropim-sulfonamida	○	○	○	○	○	○	○	○	○
Metronidazol	○	○	○	○	○	○	○	○	○
Neomicina*	○	○	○	○	○	○	○	○	○
Não uso terapêutica	○	○	○	○	○	○	○	○	○

- **Utilização ao abrigo da cascata.**

11- Selecione para cada doença qual ou quais os antibióticos, que utiliza com maior frequência:
11.4- Aparelho Músculo-esquelético

Doença / Antibiótico	Osteomielite		Fratura exposta		Pré-cirurgia		Pós-cirurgia	
	CÃO	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**	**CAO**	**GATO**
Penicilina G	○	○	○	○	○	○	○	○
Ampicilina	○	○	○	○	○	○	○	○
Amoxicilina	○	○	○	○	○	○	○	○
Amoxicilina/ Ácido clavulânico	○	○	○	○	○	○	○	○
Cefalexina	○	○	○	○	○	○	○	○
Cefovecina	○	○	○	○	○	○	○	○
Enrofloxacina	○	○	○	○	○	○	○	○
Marbofloxacina	○	○	○	○	○	○	○	○
Pradofloxacina	○	○	○	○	○	○	○	○
Doxiciclina	○	○	○	○	○	○	○	○
Gentamicina	○	○	○	○	○	○	○	○
Clindamicina	○	○	○	○	○	○	○	○
Trimetropim-sulfonamida	○	○	○	○	○	○	○	○
Metronidazol	○	○	○	○	○	○	○	○
Não uso terapêutica	○	○	○	○	○	○	○	○

11- Selecione para cada doença qual ou quais os antibióticos, que utiliza com maior frequência:
11.5- Aparelho Respiratório

Doença / Antibiótico	Infeção do trato respiratório superior		Traqueo-Bronquite Infeciosa	Complexo respiratório Felino	Pneumonia		Piotórax	
	CÃO	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**	**CÃO**	**GATO**
Penicilina G	○	○	○	○	○	○	○	○
Ampicilina	○	○	○	○	○	○	○	○
Amoxicilina	○	○	○	○	○	○	○	○
Amoxiciclina/ Ácido clavulânico	○	○	○	○	○	○	○	○
Cefalexina	○	○	○	○	○	○	○	○
Cefovecina	○	○	○	○	○	○	○	○
Enrofloxacina	○	○	○	○	○	○	○	○
Marbofloxacina	○	○	○	○	○	○	○	○
Pradofloxacina	○	○	○	○	○	○	○	○
Doxiciclina	○	○	○	○	○	○	○	○
Gentamicina	○	○	○	○	○	○	○	○
Clindamicina	○	○	○	○	○	○	○	○
Trimetropim-sulfonamida	○	○	○	○	○	○	○	○
Metronidazol	○	○	○	○	○	○	○	○
Não uso terapêutica	○	○	○	○	○	○	○	○

Annex 4 – Tables with percentage values for all disorders for dogs and cats.

	Skins disorders					
	Dogs				Cats	
	Pyoderma dog	Deep Pyoderma dog	Abscess dog	Bacterial otitis Dog	Abscess Cat	Bacterial otitis Cat
Penicilin G	2%	1%	3%	1%	3%	2%
Ampiciline	1%	1%	1%	1%	1%	2%
Amoxicillin	4%	1%	2%	1%	2%	1%
Amoxicillin-clavulanate	54%	29%	39%	34%	38%	32%
Cephalexin	23%	33%	8%	10%	6%	10%
Cefovecin	3%	4%	2%	1%	3%	3%
Enrofloxacin	5%	17%	16%	23%	23%	28%
Marbofloxacin	1%	4%	4%	10%	5%	9%
Pradofloxacin	0%	2%	3%	1%	4%	1%
Doxycyclin	1%	1%	1%	0%	1%	0%
Gentamicin	0%	0%	0%	12%	0%	9%
Clindamycin	1%	4%	5%	1%	1%	2%
Trimethopim-sulphonamide	1%	0%	0%	1%	0%	0%
Metronidazole	0%	4%	18%	1%	14%	1%
Florfenicol	0%	0%	0%	0%	0%	0%
No therapeutic	4%	0%	0%	2%	0%	2%

	Urinary tract infections								
	Dogs					Cats			
	UTI Dog	UTI complicated Dog	Pyelonephrytis Dog	Prostatitis Dog	Pyometra Dog	UTI Cat	UTI complicated Cat	Pyelonephrytis Cat	FLUTD
Penicilin G	0%	1%	1%	1%	0%	0%	1%	1%	0%
Ampiciline	0%	2%	3%	0%	2%	0%	1%	2%	0%
Amoxicillin	3%	1%	3%	2%	2%	3%	1%	3%	2%
Amoxicillin-clavulanate	35%	18%	25%	16%	34%	32%	18%	26%	20%
Cephalexin	9%	13%	13%	6%	9%	7%	13%	13%	7%
Cefovecin	3%	6%	3%	0%	2%	4%	7%	4%	3%
Enrofloxacin	34%	32%	30%	44%	26%	40%	34%	30%	26%
Marbofloxacin	7%	8%	5%	9%	3%	6%	10%	7%	3%
Pradofloxacin	1%	4%	4%	4%	1%	3%	4%	4%	1%
Doxycyclin	2%	3%	1%	1%	2%	2%	3%	1%	0%
Gentamicin	1%	1%	2%	0%	1%	1%	1%	1%	1%
Clindamycin	0%	1%	1%	1%	0%	0%	1%	1%	0%
Trimethopim-sulphonamide	4%	6%	4%	13%	2%	3%	3%	2%	1%
Metronidazole	1%	1%	1%	1%	15%	1%	1%	1%	0%
Nitrofuranoin	0%	0%	1%	1%	0%	0%	1%	0%	1%
Phosfomycin	1%	1%	0%	0%	0%	0%	0%	0%	0%
No therapeutic	1%	1%	3%	1%	3%	1%	1%	3%	35%

	Gastrointestinal tract disorders								
	Dogs					Cats			
	Acute gastritis dog	Periodontal disease dog	Chronic gastritis dog	Enteritis dog	Colitis dog	Periodontal disease cat	Chronic gastritis cat	Enteritis cat	Colitis cat
Penicilin G	1%	1%	2%	2%	1%	1%	2%	1%	1%
Ampiciline	1%	1%	4%	6%	3%	1%	3%	6%	2%
Amoxicillin	6%	4%	6%	4%	3%	4%	7%	4%	3%
Amoxicillin-clavulanate	29%	30%	18%	19%	17%	29%	18%	22%	18%
Cephalexin	4%	1%	3%	1%	1%	3%	3%	1%	1%
Cefovecin	7%	3%	0%	1%	1%	8%	0%	1%	2%
Enrofloxacin	9%	7%	4%	8%	6%	6%	3%	8%	6%
Marbofloxacin	0%	1%	0%	1%	1%	1%	0%	1%	1%
Pradofloxacin	1%	3%	0%	0%	0%	3%	0%	0%	0%
Doxycyclin	4%	1%	1%	1%	1%	2%	0%	1%	1%
Gentamicin	1%	1%	2%	2%	1%	1%	1%	1%	1%
Clindamycin	4%	5%	0%	1%	1%	3%	0%	0%	1%
Trimethopim-sulphonamide	1%	1%	2%	9%	8%	1%	3%	6%	6%
Metronidazole	27%	39%	19%	39%	46%	35%	17%	40%	46%
Neomicin	1%	0%	0%	1%	3%	0%	0%	1%	3%
No therapeutic	7%	3%	40%	5%	7%	3%	42%	6%	7%

	Respiratory tract diseases							
	Dogs				Cats			
	Canine upper respiratory tract disease	Tracheobronchitis dog	Pneumonia dog	Pyothorax dog	Feline upper respiratory tract disease	Feline respiratory complex	Pneumonia cat	Pyothorax cat
Penicilin G	1%	1%	2%	2%	1%	1%	2%	2%
Ampiciline	1%	1%	3%	2%	1%	1%	2%	1%
Amoxicillin	4%	5%	1%	1%	4%	3%	2%	1%
Amoxicillin-clavulanate	45%	36%	29%	25%	40%	32%	30%	25%
Cephalexin	8%	8%	12%	12%	8%	7%	12%	12%
Cefovecin	1%	0%	0%	0%	1%	3%	1%	0%
Enrofloxacin	15%	10%	24%	23%	17%	15%	24%	22%
Marbofloxacin	3%	1%	6%	5%	2%	2%	6%	5%
Pradofloxacin	1%	1%	2%	1%	1%	1%	3%	1%
Doxycyclin	16%	29%	9%	2%	21%	28%	12%	2%
Gentamicin	1%	1%	2%	3%	1%	1%	1%	2%
Clindamycin	0%	0%	1%	1%	1%	2%	1%	2%
Trimethopim-sulphonamide	2%	1%	3%	2%	0%	0%	2%	2%
Metronidazole	0%	0%	5%	17%	0%	0%	5%	17%
No therapeutic	2%	5%	0%	2%	2%	5%	1%	2%

	Orthopedic conditions					
	Dogs			Cats		
	Osteomyelitis dog	Exposure fracture dog	Pre-surgery dog	Osteomyelitis cat	Exposure fracture cat	Pre-surgery cat
Penicilin G	2%	2%	5%	1%	2%	5%
Ampiciline	1%	1%	2%	1%	1%	2%
Amoxicillin	1%	1%	4%	1%	1%	5%
Amoxicillin-clavulanate	14%	30%	42%	14%	30%	42%
Cephalexin	23%	22%	16%	23%	21%	15%
Cefovecin	3%	2%	3%	4%	2%	3%
Enrofloxacin	13%	15%	4%	15%	15%	4%
Marbofloxacin	2%	1%	1%	2%	2%	2%
Pradofloxacin	1%	1%	0%	1%	1%	0%
Doxycyclin	1%	0%	0%	0%	0%	0%
Gentamicin	2%	1%	1%	2%	1%	1%
Clindamycin	25%	9%	3%	22%	8%	2%
Trimethopim-sulphonamide	2%	2%	0%	1%	2%	0%
Metronidazole	9%	14%	1%	9%	14%	1%
No therapeutic	3%	1%	19%	4%	1%	19%

Annex 5 - Abstract of the oral communication at the OMV Conference 2012

Abstract of the Oral presentation at the OMV Conference 2012

Etiologia e Detecção de estirpes multiresistentes na infecção do trato urinário em cães e gatos em Lisboa, Portugal.

D. Saial[1], A. Belas[1], M. Centeno[1], N. Couto[1], C. Pomba[1].

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O objectivo deste estudo foi determinar a etiologia da ITU na comunidade e a frequência de agentes patogénicos multirresistentes na região de Lisboa.

No presente estudo foram isoladas 136 bactérias uropatogénicas, em cães (*n=108*) e em gatos (*n=28*), durante o período de Janeiro de 2009 até Dezembro de 2011 no Hospital Veterinário da Universidade Técnica de Lisboa e em Hospitais e Clínicas privadas na área de Lisboa. Os isolados foram identificados por sistemas bioquímicos. Os métodos de difusão de disco e painéis de microdiluição DADE MicroScan foram utilizados para os testes de susceptibilidade aos antimicrobianos.

Em cães, *Escherichia coli* foi o agente uropatogénico mais frequente (52%), seguido de *Staphylococcus* spp. (14%), *Proteus* spp. (13%), *Enterococcus* spp. (11%), *Pseudomonas* spp. (4%), *Klebsiella* spp. (4%), e outros (2%). Em gatos, *Escherichia coli* foi também o mais frequente (46%), seguido de *Staphylococcus* spp. (11%), *Enterococcus* spp. (11%), *Enterobacter* spp. (11%), *Pseudomonas* spp. (4%), *Proteus* spp. (4%) e outros (13%). Detectaram-se duas estirpes de *E. coli* produtoras de cefalosporinases (pAmpC) e uma enzima β-lactamase de espectro alargado (ESBL). Um isolado de *Acinetobacter baumannii* foi identificado como produtor de pAmpC. Dois isolados de *Enterococcus* spp. susceptíveis à vancomicina exibiram um alto nível de resistência à gentamicina (HLGR), sendo um deles resistente à ampicilina, fármaco de eleição para a terapêutica da infecção por *Enterococcus*. Dois staphylococci demonstraram resistência à oxacilina e multirresistência a mais de 3 classes de antimicrobianos diferentes (*Staphylococcus pseudintermedius* meticilina-resistente – MRSP).

A detecção de estirpes gram-negativas produtoras de pAmpC, ESBL e estirpes gram-positivas como MRSP e *Enterococcus* com resistência à ampicilina e HLGR é extremamente preocupante e com consequências inerentes de limitação terapêutica. A presença de bactérias uropatogénicas multirresistentes constitui não só um problema de saúde animal mas também de saúde pública.

Annex 6 - Poster presented at the International Meeting on Emerging Diseases and Surveillance - IMED 2013.

International Meeting on Emerging Diseases and Surveillance

Vienna, Austria • February 15–18, 2013

Co-sponsored by

ProMED-mail, the Program for Monitoring Emerging Diseases
EcoHealth Alliance
European Centre for Disease Prevention and Control (ECDC)
European Society of Clinical Microbiology and Infectious Diseases (ESCMID)
Food and Agriculture Organization of the United Nations (FAO)
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the whole period studied, CHA was more efficient than BAC. Moreover, CHA was almost as efficient at 5 minutes as it was at 60 minutes. Furthermore, to achieve the same efficiency, a higher concentration of BAC or a longer exposure to the biocide was necessary, when compared to CHA.

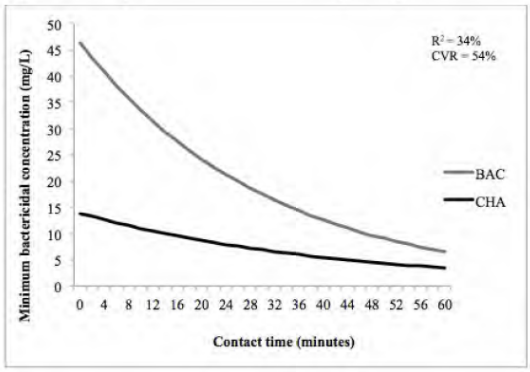

Figure 1. Activity of CHA and BAC over time (minutes).

Conclusion: Overall CHA was better than BAC at 20ºC, since less time and a lower concentration was needed to kill all MRS. In order to use BAC for decolonization we would have to use a much higher concentration or increase the exposure time to achieve killing of MRS. Our results suggest that CHA may be the best antiseptic to decolonize horses carrying MRS when entering a veterinary hospital.

21.023 Detection of bacteria resistant to critically important antimicrobials from lower urinary tract infections in cats and dogs from Portugal

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Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa, Lisbon, Portugal

Background: Cephalosporins, fluoroquinolones and aminoglycosides are classified as critically important antimicrobials by the OIE and also meet the WHO criteria 1 (sole therapy or one of few alternatives to treat serious human disease) and 2 (used to treat diseases caused by organisms that may be transmitted via non-human sources or that may acquire resistance genes from non-human sources).

Objectives: To detect critically important antimicrobial resistant bacteria among strains isolated from lower urinary tract infections originated from cats and dogs in Portugal.

Methods and Materials: Eight hundred and forty five uropathogenic bacteria were isolated from dogs (n=1160) and cats (n=553), between January1999 and November 2012, at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine and at veterinary private practices in the Lisbon area. Isolates were identified using standard commercial systems. Susceptibility testing was performed using the disk diffusion and broth microdilution methods. Extended-spectrum β-lactamases (ESBL) production was screened by double-disk synergy test. The ESBL, plasmid-mediated *ampC* and *mecA* genes were detected by PCR and gene enzymes were sequenced.

Results: Overall, among Enterobacteriaceae resistance to cephalosporins was 7.1%, 22% to fluoroquinolones (FQ) and 13.3% to aminoglycosides (AG). Thirty-three *Enterobacteriaceae* isolates were resistant to cefoxitin but only 12 produced plasmid-mediated cephalosporinases (11 bla_{CMY-2} and 1 bla_{DHA-1}). Seven *E. coli* isolates produced ESBL (3 $bla_{CTX-M-15}$, 3 $bla_{CTX-M-32}$ and 1 $bla_{CTX-M-14}$) and were also resistant to ciprofloxacin. Eight staphylococci exhibited resistance to oxacilin (6.7%) and had the *mecA* gene (2 *S. aureus*, 3 *S. pseudintermedius* and 3 *S. epidermidis*). Staphyloccoci were 10.1% resistant to FQ and 8.5% to AG. Seven *Enterococcus* spp. were resistant to ampicillin and 3 strains also exhibited high-level-gentamicin-resistance.

Conclusion: These results demonstrate that the emergence of resistance to critically important antimicrobials among uropathogens from companion animals is a concerning fact. Furthermore the detection of uropathogens with antimicrobial resistance is not only an animal health issue but also a matter of public health, since companion animals may act as reservoirs of antimicrobial resistant bacteria or resistance genes for humans.

21.024 Trends in the prevalence of methicillin resistance in staphylococci isolated from companion animals

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Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa, Lisbon, Portugal

Background: In recent years, methicillin resistance has emerged in staphylococci isolates from animals, turning it to a serious public health issue. These days not only coagulase-positive staphylococci, but also coagulase-negative, are recognized as significant clinical pathogens, in which the *mecA* gene has been detected.

Objectives: To detect methicillin-resistant staphylococci (MRS) isolates from infections in companion animals in Portugal.

Methods and Materials: Six hundred and nine staphylococci were isolated from sick horses, dogs, cats and rabbits, between January1999 and November 2012, at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine and at veterinary private practices in the Lisbon area. Isolates were identified at the species level using BBL Crystal™ Gram Positive Identification System and *nuc*-specific PCR. Susceptibility testing was performed using both the disk diffusion and broth microdilution methods, according to CLSI guidelines, and methicillin-resistance was confirmed by PCR amplification of the *mecA* gene. MRS isolates were subjected to SCC*mec*-typing.

Results: Forty-six staphylococci exhibited resistance to oxacilin and had the *mecA* gene (6 *Staphylococcus aureus*, 19 *Staphylococcus pseudintermedius*, 11 *Staphylococcus epidermidis*, 3 *Staphylococcus haemolyticus*, 1 *Staphylococcus sciuri* and 6 were *Staphylococcus* spp.). The first MRS detected was in 2001 and was a *S. aureus* strain. Since then the prevalence of MRS infections has increased and in 2012 MRS isolates accounted for 51% of the staphylococci strains. All MRS isolates were resistant to at least one more antimicrobial class other than β-lactam (fluoroquinolones or aminoglycosides). None of the isolates was resistant to vancomycin, quinupristin/dalfopristin or linezolid, drugs of choice for the treatment of MRS human infections. SCC*mec*-typing revealed type III (*S. pseudintermedius*, *S. epidermidis* and *S. sciuri*), type IV (*S. aureus* and *S. epidermidis*), type V (*S. pseudintermedius* and *S. haemolyticus*) and type VI (*S. epidermidis*) cassettes.

Conclusion: The prevalence of MRS infection in our companion animal population is increasing. This may compromise antimicrobial therapy and can become life threatening if not well diagnosed. Moreover, the presence of these MRS in sick animals highlights the importance of horizontal transfer of different SCC*mec* elements and might favor the transmission of resistance genes from animals to humans.

21.025 Isolation and characterization of anti-*Pythium insidiosum* compounds from *Pseudomonas stutzeri* isolated from aquatic environment

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Background: Pythiosis is a granulomatous disease which mostly found in tropical, subtropical and temperate regions. Most cases of pythiosis have been found in animals and humans caused by a fungus-like *Pythium insidiosum*. Failure of drug treatment of pythiosis combined with a high rate of morbidity and mortality have drawn attention to search for new antifungal agents.

Annex 7 – Abstract approved for Oral Communication at the ESCMID – Conference on *E. coli.*

Different epidemiology of *bla*$_{CMY-2}$ plasmids among clinical *Escherichia coli* from companion animals in Portugal and Denmark

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Objective: The aim of this study was to compare the epidemiology of CMY-2 β-lactamase-encoding genes among clinical *E. coli* from dogs and cats in two European countries with different antibiotic use practices.

Methods: We characterised the *bla*$_{CMY-2}$-harbouring plasmids of eight and five *E. coli* collected in 2000-2012 at the veterinary diagnostic laboratories of the Technical University of Lisbon and the University of Copenhagen, respectively. Plasmids harbouring *bla*$_{CMY-2}$ were transferred into GeneHog *E. coli* by electroporation and typed by S1 endonuclease pulsed-field gel electrophoresis, PCR-based replicon typing, plasmid multilocus sequence typing (pMLST) and antimicrobial susceptibility testing of transformants to detect co-transfer of antimicrobial resistance.

Results: The eight *bla*$_{CMY-2}$-harbouring plasmids from Portugal belonged to two distinct incompatibility groups: IncFII/F2:A-:B- of ca. 78 Kb (n=7), and IncA/C of ca. 140 Kb (n=1). IncFII plasmids encoded additional resistance to ciprofloxacin, gentamicin, sulphametoxazole-trimethoprim and tetracyclines. The five plasmids from Denmark were all IncI1 ranging from 55 to 104 Kb, were classified as ST2 (n=2) and non-typeable (n=3) by pMLST. The Danish plasmids did not co-transfer resistance to non-β-lactam antimicrobials.

Conclusion: Spread of *bla*$_{CMY-2}$ was associated with different plasmid types among isolates from the two countries, being mainly driven by IncFII encoding a multidrug resistance phenotype in Portugal and by IncI1 not associated with multidrug resistance in Denmark. Since the plasmid types detected among companion animals partially overlap with the types found in humans in the respective countries, further studies are needed to determine whether transmission of *bla*$_{CMY-2}$ from dogs and cats represents a zoonotic risk.